Spectrin Oxidation Correlates With Membrane Vesiculation in Stored RBCs

By G.M. Wagner, D.T-Y. Chiu, J-H. Qju, R.H. Heath, and B.H. Lubin

An increase in spectrin oxidation in a variety of erythrocytes displaying a tendency to vesiculate has been previously described. To explore this relationship in more detail, we have studied blood stored in citrate-phosphate-dextrose-adenine under blood bank conditions because, in this system, vesiculation occurs slowly. Vesiculation was quantitated by measuring acetylcholinesterase release, and the extent of spectrin oxidation was detected by using thiol-disulfide exchange chromatography. A strong correlation (r = .92) was found between the extent of spectrin oxidation and vesiculation when blood from five donors was analyzed at weekly intervals during storage. This strongly suggests that spectrin oxidation plays a role in the formation of spectrin-free vesicles, thereby limiting the shelf life of stored blood.

THE MECHANISM BY which the RBC membrane vesiculates is of particular importance in blood banking since posttransfusion viability of RBC appears to be limited by reduction in cell surface area.[1-3] Shedding of spectrin-free vesicles from echinocytic erythrocytes leads to the formation of dense, poorly deformable spherocytes that are quickly removed from the circulation.[2,3] In the past, several factors including cellular levels of adenosine triphosphate (ATP),[4-6] the behavior of the calcium ion,[7,8] and the structure and function of membrane proteins[9,10] have been extensively analyzed in stored RBC, but none has been found clearly to correlate with the development of spherocytes. Recently, Wolfe et al have demonstrated that a defect in the spectrin-actin–protein 4.1 interaction developed during storage and that normal protein interactions were restored with reducing agents.[11]

We have previously examined several red cell disorders (sickle cell anemia, hemoglobin H disease, and hereditary spherocytosis) and in vitro manipulations (ATP depletion, Ca^{+2} loading, and diamide treatment) in which vesiculation occurs.[12] In each case, membrane protein oxidation could be detected[12] by thiol-disulfide exchange chromatography.[13] Therefore, as will be described, we quantitated spectrin oxidation as RBC slowly vesiculate during storage in citrate-phosphate-dextrose-adenine (CPD-A). Under these conditions, there was a striking correlation between spectrin oxidation and vesiculation.

MATERIALS AND METHODS

Determination of the Extent of Vesiculation

Acetylcholinesterase determination. The activity of AChE was assayed as described by Ellman et al.[14] This assay was used as a measure of extent of red cell vesiculation as initially outlined by Ott et al.[15]

Measurement of phospholipid release. Vesiculation of washed fresh, normal erythrocytes (hematocrit, 20%) was induced by incubation in 10 mmol/L Tris-buffered saline (TBS), pH 7.4, containing 1 mmol/L $CaCl_2$ and 2 μmol/L A23187, the calcium ionophore. This is a slight modification of the method of Allan and Thomas[16] using a lower concentration of ionophore to slow the rate of vesiculation. To stop the vesiculation process, samples were removed at four-minute intervals and pipetted into 100 mmol/L EDTA to yield a final concentration of 3 mmol/L. The red cell count of the cell suspension was determined in a Coulter counter (Coulter Electronics, Hialeah, FL). The phospholipids in 200 μL of cell suspension were extracted by the method of Rose and Oklander,[17] and the phospholipids in 1 mL of supernatant were extracted by the method of Bligh and Dyer.[18] Inorganic phosphorus in these samples was measured after sulfuric acid hydrolysis.[19]

ATP Measurement

ATP was extracted from RBC with perchloric acid by the method of Beutler[20] and was quantitated by using a commercially available luciferin/luciferase assay (Packard, Downers Grove, IL).

Osmotic Gradient Ektacytometry

Osmotic gradient ektacytometry was performed by the method of Clark et al.[21] This technique measures the deformability index (DI) of erythrocytes over a continuous osmotic gradient.

Thiol-Disulfide Exchange Chromatography

Thiol-disulfide exchange chromatography was performed with the method of Rank et al.[13] Theoretically, when this technique is used, proteins with no available sulfhydryl groups (filtrate fraction) do not bind to the resin and can be separated from proteins containing sulfhydryl groups available for binding. The bound proteins can be eluted with the reducing agent, β-mercaptoethanol (gel fraction). When the original solubilized membranes, the filtrate (oxidized), and the gel (reduced) fractions are analyzed by reducing Laemmli sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis,[22] it is apparent that after chromatography band 7 is present only in the filtrate fraction (Fig 1). Therefore, the extent of spectrin oxidation in a given sample can be expressed in terms of the spectrin:band 7 ratio determined by densitometric scanning of the gel. The ratio of band 7 to band 3 in the original membranes not subjected to chromatography was determined to assess the stability of band 7 during storage.

Determination of the Sulfhydryl Content of the Oxidized Fraction by Using Amino Acid Analysis

Ghosts were prepared from erythrocytes that had been incubated at 37°C for 48 hours in TBS, pH 7.4, containing penicillin and streptomycin, to induce protein oxidation by ATP depletion.[23] Thiol-disulfide chromatography was performed on these ghosts and the

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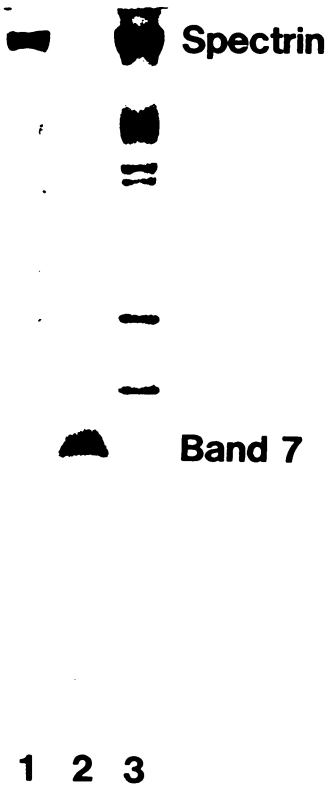

Fig 1. Laemmli SDS–polyacrylamide gel electrophoresis of the thiol-disulfide gel (reduced) fraction (lane 1), filtrate (oxidized) fraction (lane 2), and original unchromatographed ghost membranes (lane 3). Note that band 7 is found in the oxidized but not the reduced fraction.

oxidized fraction (consisting predominantly of spectrin, hemoglobin, and proteolytic fragments) obtained. The free sulfhydryl content of the oxidized proteins was measured by labeling them with iodoacetic acid, a reagent that reacts with free sulfhydryl groups to form carboxymethyl cysteine.[24] Initially, the oxidized fraction was divided into two aliquots, one of which was immediately reduced in 2 mmol/L dithiotheitol (DTT). The oxidized aliquot was dialyzed overnight at room temperature against 8 mmol/L urea, 0.1 mol/L Tris, pH 8.5; the reduced aliquot was dialyzed against the same buffer containing 2 mmol/L DTT. After dialysis the samples were placed in screw top tubes, flushed with N_2, sealed, and incubated at 30°C for 30 minutes. Then 0.5 mol/L iodoacetic acid was added to a final concentration of 10 mmol/L and the samples incubated in the dark for an additional 30 minutes at 30°C. The reaction was terminated by adding one drop of β-mercaptoethanol. The fractions were dialyzed overnight against 2 mol/L urea, 50 mmol/L Tris, pH 8.5, at 4°C and then for 48 hours against water. The samples were acid hydrolyzed, and amino acid analysis was performed.[25]

Blood Storage

Four units of blood obtained from four normal donors and two units, drawn on separate occasions from a woman therapeutically phlebotomized for polycythemia secondary to chronic lung disease, were stored in CPD-A under standard blood bank conditions for 8 weeks. Samples were removed at weekly intervals for AChE determinations to quantitate the extent of vesiculation occurring during storage, ATP extraction, ektacytometry, and thiol-disulfide chromatography.

RESULTS

AChE Release Is Linearly Correlated With Phospholipid Release

The initial goal of these studies was to demonstrate the validity of the methods used to quantitate both vesiculation and spectrin oxidation. The extent of vesiculation during storage was quantitated by measuring the percentage of the total whole blood AChE that was present in the plasma. However, this percentage does not equal that of phospholipid released because AChE is concentrated in vesicles.[26] Because storage vesiculation takes place in lipid-rich plasma, determining the nature of the correlation between AChE release and phospholipid release was not feasible in this system. Therefore to examine this problem, we induced vesiculation in fresh washed erythrocytes by calcium loading and found that AChE release is linearly related to phospholipid release (Fig 2). The calcium-loading system was used in these experiments because maximum vesiculation could be achieved in 60 minutes, the vesiculation process could be halted instantly by the addition of EDTA, and most importantly, there was no exogenous phospholipid in the incubation buffer. A similar linear relationship between AChE and phospholipid release was found when vesiculation due to ATP depletion was studied (data not shown). Since such a relationship is present in two very different forms of the vesiculation process,[23] we feel that a linear relation between AChE release and phospholipid release during storage vesiculation is highly likely as well. Therefore in all storage experiments we measured AChE release to quantitate vesiculation.

Measurement of Spectrin Oxidation

To quantitate the extent of spectrin oxidation, we performed thiol-disulfide exchange chromatography on solubilized ghost membranes to separate oxidized and reduced proteins. The original ghost membranes and both column fractions were analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions. The extent of spectrin oxidation was quantitated by measuring the ratio of spectrin

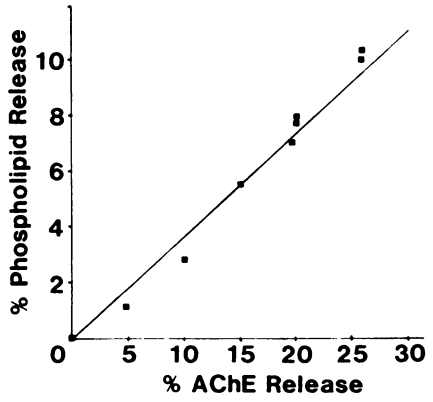

Fig 2. Percentage of AChE released during calcium loading v percentage of phospholipid release (a representative experiment).

to band 7 in the oxidized fraction. To ensure that changes in this ratio during storage were not due to changes in band 7, the band 7:band 3 ratio in the original membrane samples was measured. In all samples this ratio remained constant during storage (0.10 ± 0.02), thus indicating that there was no significant loss of band 7 and that changes in the spectrin:band 7 ratio were due to changes in the amount of oxidized spectrin.

Amino acid analysis experiments indicate that proteins in the filtrate fraction are approximately two-thirds oxidized. An aliquot of oxidized proteins treated with iodoacetic acid contained only 34.5% as much carboxymethyl cysteine as an aliquot reduced before treatment. The oxidized fraction analyzed in this way was a mixture of proteins, so the exact extent of spectrin oxidation cannot be stated. Nevertheless, it is reasonable to assume that the proteins in the oxidized fraction have been oxidized to a similar degree. Therefore, the thiol-disulfide chromatographic method allows us to quantitate spectrin that has been severely oxidized.

Vesiculation of Erythrocytes During Storage

We have previously demonstrated increased spectrin oxidation in a variety of conditions known to be associated with red cell vesiculation.[12] To examine this phenomenon in more detail we have selected an experimental system in which vesiculation occurs slowly, namely, in blood stored under blood bank conditions. Figure 3 shows AChE release during storage of units of whole blood from four different donors. There is individual variation in the rate and extent of vesiculation. This is consistent with previous observations that there are differences in the storage stability of blood from different donors.[2,3] An ektacytometer was used to determine the deformability of a "rapid" and a "slow" vesiculating unit before and after 6 weeks of storage. The deformability pattern of the rapid unit showed a decrease in maximal deformability during storage consistent with membrane loss, whereas the pattern of the slow unit was only slightly changed (Fig 4).

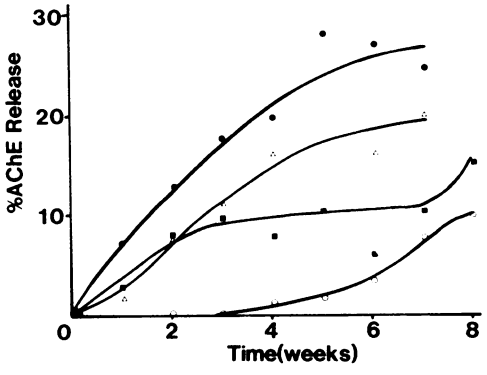

Fig 3. Percentage of AChE released from 4 units of stored RBC *v* time. Donor 1, ●—●; donor 2, △—△; donor 3, ■—■; donor 4, ○—○. Note: Units from donor 1 were analyzed on two separate occasions and gave similar results each time. Blood from a fifth donor behaved in a manner virtually identical to donor 1, but for the sake of clarity, the data are not shown.

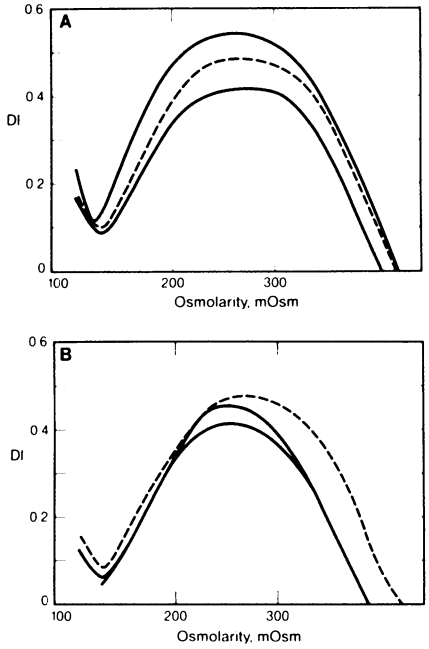

Fig 4. DI as a function of osmolarity measured by ektacytometry. The osmolarity scale is not linear. (A) A rapidly vesiculating blood sample (donor 5). (B) A slowly vesiculating blood sample (donor 4). In each case the upper solid line is the pattern obtained from a fresh sample, and the lower solid line is the pattern obtained after 6 weeks of storage. The dashed line is the pattern of fresh blood from a normal donor analyzed with each sample to serve as a reference point.

Vesiculation During Storage Does Not Correlate With ATP Levels

It is likely that membrane vesiculation and the subsequent loss of deformability is the elusive "storage lesion" that limits the shelf life of stored blood. Therefore, we followed ATP levels and spectrin oxidation during storage to determine whether changes in either of these parameters correlated with vesiculation. Although energy depletion induces vesiculation of erythrocytes in vitro[27] vesiculation during storage did not appear to be related primarily to decreased levels of

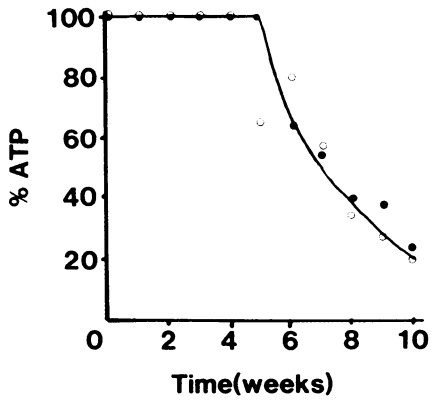

Fig 5. Percentage of the initial ATP level *v* time for donor 1, the most actively vesiculating sample, on two separate occasions. Similar results were obtained on blood from the other donors.

ATP. In most units, vesiculation during storage began immediately (Fig 3) and was active while cellular ATP levels were high (Fig 5).

Vesiculation During Storage Correlates With Spectrin Oxidation

Although vesiculation of blood stored in CDP-A was unrelated to ATP depletion, thiol-disulfide chromatography performed on stored blood samples provided evidence that vesiculation and spectrin oxidation are related. Representative electrophoretic patterns of oxidized chromatography fractions are shown in Fig 6A. It can be seen that the spectrin band of the sample stored for 6 weeks is considerably more dense than that seen in a fresh sample from the same donor. In Fig 6B the change in the spectrin:band 7 ratio with time for 4 units is shown. The shape of the curve for each unit is remarkably similar to its corresponding curve in Fig 3. In Fig 7, the spectrin:band 7 ratio is plotted against the percentage of AChE release (vesiculation). Not only is there excellent correlation between the extent of spectrin oxidation and the extent of vesiculation (r = .92), but points derived from all units appear to fall on the same line.

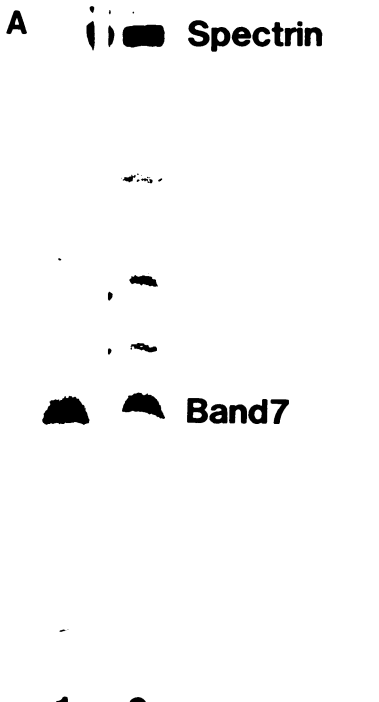

Fig 6. (A) Representative SDS gels of oxidized fractions from donor 1 obtained during thiol-disulfide exchange chromatography. Lane 1, fresh sample. Lane 2, sample after 6 weeks' storage. (B) Spectrin:Band 7 ratio as a function of time. Donor 1, ●—●; donor 2, △—△; Donor 3, ■—■; donor 4, ○—○. The symbols for each donor correspond to those used in Fig 3.

DISCUSSION

The occurrence of membrane vesiculation and the resultant formation of poorly deformable spherocytes is likely to be the cause of the disappointingly short shelf life of stored blood. We have shown that during storage, oxidation of spectrin is occurring and that this process correlates well with vesiculation. This observation complements those of Wolfe et al who found that during storage a reducing agent correctable defect in spectrin:actin:protein 4.1 interaction occurs.[11] Becker et al demonstrated that oxidation of as few as one spectrin sulfhydryl group can produce this defect in a purified protein system and that oxidation of two sulfhydryl groups produces structural alterations in spectrin binding to inside-out vesicles.[28] Thiol-disulfide chromatography detects only severely oxidized proteins that may represent only a small fraction of those that have undergone some degree of oxidative damage. Although mildly oxidized spectrin may be involved in the vesiculation process, vesiculation clearly correlates with the amount of severely oxidized spectrin.

The exact mechanism by which vesiculation occurs is unknown, but it is conceivable that, as spectrin is oxidatively damaged in a small area of the membrane, localized spectrin-lipid interactions are disrupted, and the affected membrane pinches off as a spectrin-depleted free vesicle. Many such events occurring during the storage period would ultimately produce a rigid spherocyte. Why metabolically intact RBC under storage conditions are more prone to vesiculate and become spherocytes than cells in vivo is unknown. Perhaps in vivo the red cell itself has mechanisms for repair of oxidative damage to membrane proteins that are not functional during storage at 4°C, eg, ATP-dependent proteases,[29] or perhaps the reticuloendothelial system is capable of removing damaged membrane in a manner that is less

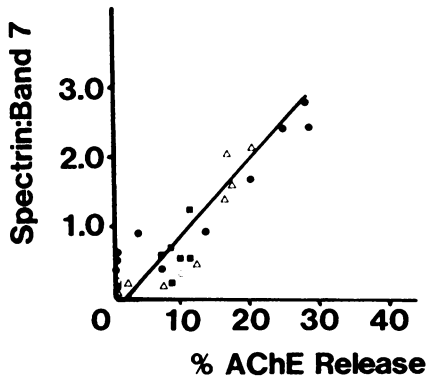

Fig 7. Spectrin:band 7 ratio as a function of the percentage of AChE release. The different symbols represent points derived from different units of blood and correspond to those used in Figs 3 and 6 (r = .92).

disruptive to the cell than vesiculation. In any case, our observations support the concept that oxidation of membrane components is involved in the storage lesion and suggest that the use of antioxidants or agents that inhibit vesiculation might be useful for prolonging the shelf life or improving the quality of blood used for transfusion.

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