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Antioxidant Status and Susceptibility of Sickle Erythrocytes to Oxidative and Osmotic Stress

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The purpose of this study was to determine if differences in antioxidant status between the red blood cells (RBCs) of sickle cell anemia (SCA) patients and controls are responsible for the differential responses to oxidative and osmotic stress-induced hemolysis. Susceptibility to hemolysis was examined by incubating oxygenated and deoxygenated RBCs at 37°C with 73 mM 2,2' azobis (2-amidinopropane) HCl (AAPH), a peroxyl radical generator, for up to 3.5 hours. The ability of RBCs to maintain membrane integrity under osmotic stress was determined over a range of diluted saline-phosphate buffer. Sickled RBCs showed a lesser degree of AAPH-induced hemolysis than control groups and were more resistant to osmotic stress-induced hemolysis. SCA patients had higher levels of RBC vitamin E and RBC lipids, but lower RBC GSH, plasma lipids and plasma carotenes than those of the hospital controls. No significant differences were observed in the levels of retinol, vitamin C, vitamin E, MDA and conjugated dienes in plasma, or the levels of MDA and conjugated dienes in RBCs. The results obtained suggest that the differences in antioxidant status between sickled RBCs and controls do not appear to be responsible for their different susceptibility to oxidative or osmotic stress-induced hemolysis observed.

INTRODUCTION

Sickle cell anemia (SCA) is a chronic hemolytic anemia resulting from a single gene mutation in the globin chain of hemoglobin.[1] The abnormal hemoglobin produced by individuals with this mutation undergoes polymerization when deoxygenated, causing the formation of distorted, 'sickle' shaped erythrocytes. Sickle cell disease is characterized by vascular obstruction, infection, anemia, hemolysis and shortened red blood cell (RBC) lifespan.[2] Despite considerable research, the mechanisms by which these complications develop have yet to be elucidated.

Free radical induced peroxidative membrane damage has been implicated in the pathogenesis of many injury and disease states.[3–5] Oxidative damage to RBC membranes can lead to increased membrane rigidity, decreased deformability, altered membrane permeability and immune

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recognition, increased adherence to endothelial cells, and increased cell aggregation and lysis.[6–8] Any of which could contribute to the complications developed in SCA. Sickle RBCs have been shown to be more susceptible than normal RBCs to oxidizing agents.[8–13] This increased susceptibility of sickle RBCs to oxidative stress has been attributed to abnormalities associated with the unstable hemoglobin, antioxidant deficiencies or changes in RBC membrane structure following sickling.[8–16]

Patients with SCA or sickle cell trait have been reported to be low in many antioxidant systems, including vitamin E, vitamin C, carotenoids, glutathione (GSH), superoxide dismutase, catalase, GSH peroxidase and glucose 6-phosphate dehydrogenase.[9,10,15–21] Inadequate RBC antioxidant capabilities are linked to an increased susceptibility of RBCs to peroxidative and hemolytic stress. However, reports on the antioxidant status of sickle cell patients are inconsistent. The plasma levels of vitamin E, for example, have been reported to be both lower than controls[9,15,16] and no different.[17] Also, whether inadequate antioxidant status leads to the development of complications characteristics of SCA remains unclear. The goal of this study was, therefore, to determine if differences in antioxidant status between sickle and control RBCs contribute to their differential responses to oxidative and osmotic stress-induced hemolysis.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol and hexane were purchased from Fisher Scientific, Cincinnati, OH and EM Science, Gibbstown, NJ, respectively. 95% Ethanol was obtained from Midwest Grain Products of Illinois, Pekin, IL. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences, Inc., Warrenton, PA. 1,1,3,3-tetramethoxy-propane (TMP), 2-thiobarbituric acid, butylated hydroxytoluene (BHT), beta-carotene and ascorbic acid were purchased from Sigma Chemical Company, St. Louis, MO. Tetrabutyl ammonium dihydrogen phosphate was obtained came from Aldrich Chemicals, Milwaukee, WI, and isobutyl alcohol from Mallinckrodt Chemicals, St. Louis, MO. Alphatocopherol was a gift from Henkel Corp., Minneapolis, MN.

Blood Preparation

Blood samples were obtained from 49 sickle cell anemia patients and 49 control subjects. SCA patients were identified by electrophoresis of HbS and by family history studies. There were 38 females and 11 males ranging in age from 21–62 years. The controls, 16 females and 33 males, ranged in age from 35–62 years. Controls were healthy, non-anemic and had no underlying medical problems. Thirty two SCA patients received nutritional supplements which included folic acid and up to 400 IU vitamin E per day as part of their treatment regimen. The remaining 17 sickle cell patients and all controls received no nutritional supplements.

Aliquots of whole blood were used to measure reduced glutathione (GSH) and vitamin C (ascorbic acid). Erythrocytes and plasma were separated by centrifugation at 2000 rpm for 5 minutes and erythrocytes were washed twice with saline-phosphate buffer (0.175 M, pH 7.4). Vitamin E, malondialdehyde (MDA), conjugated dienes and total lipids were measured in plasma and washed erythrocytes. Total carotenes and retinol were measured in plasma. Susceptibility to free radical induced hemolysis was determined in washed erythrocytes.

Susceptibility to Peroxyl Radical Induced Hemolysis

The susceptibility of RBCs to peroxyl radical induced hemolysis was studied by incubating with AAPH, which generates peroxyl radicals at

a constant rate when incubated at 37°C.[22] Washed sickle and control RBCs were diluted to a hematocrit of 10% with 0.125 M NaCl in 0.01 M phosphate buffer, pH 7.4, then mixed with an equal volume of 73 mM AAPH, 0.05 M NaCl in 0.01 M phosphate buffer, pH 7.4.[23] The RBC suspensions were then incubated at 37°C in a shaking water bath under either oxygenated or deoxygenated (nitrogen atmosphere) conditions. Aliquots of the RBC suspension, taken at 1.5, 2.5 and 3.5 hours of incubation, were diluted with saline-phosphate buffer, pH 7.4, or distilled water for measuring the degree of hemolysis based on the absorption of hemoglobin at 415 nm.

Osmotic Fragility

Washed erythrocytes were diluted with saline-phosphate buffer to 5% hematocrit. Aliquots of the RBC suspension were added to a series of test tubes containing diluted saline-phosphate buffer, pH 7.4 (26–32%). The test tubes were centrifuged and the supernatants were measured for the degree of hemolysis.

Biochemical Determinations

Vitamin E was measured according to the HPLC procedure of Hatam and Kayden[24] with fluorescence detection. Retinol was measured by the HPLC method of Collins and Chow.[25] MDA was determined by the HPLC method of Tatum *et al.*[26] GSH was measured spectrophotometrically by the method of Sedlack and Lindsay.[27] Conjugated dienes were measured spectrophotometrically as described by Recknagel and Goshal.[28] Vitamin C was determined by reaction with dinitrophenylhydrazine according to the method of Omaye *et al.*[29] Total carotenes were determined by measuring the absorbance of a hexane extract of plasma at 450 nm. Total lipids were measured by the method of Chiang *et al.*[30]

Data obtained were analyzed using ANOVA and paired T-tests.

RESULTS

Susceptibility to AAPH-Induced Hemolysis

The rate of AAPH-induced hemolysis in oxygenated and deoxygenated RBCs is shown in Figure 1. Sickle erythrocytes were more resistant than controls to hemolytic stress induced by peroxyl radicals generated from AAPH under either oxygenated or deoxygenated conditions employed. Following 3½ hours of incubation, oxygenated sickle erythrocytes were, on average, 52.5% hemolyzed while control erythrocytes were 87.3% hemolyzed (Figure 1A). Incubation following

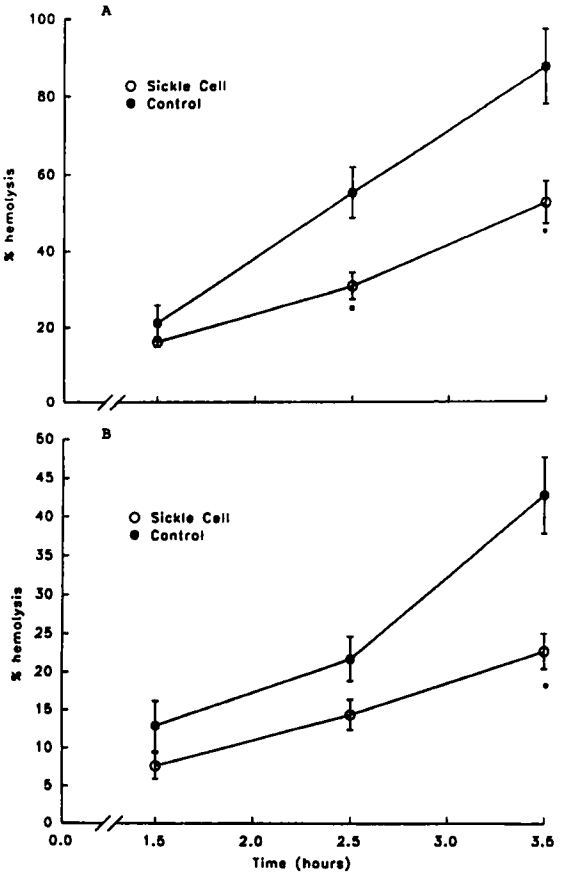

FIGURE 1 AAPH induced hemolysis in oxygenated (1A) and deoxygenerated (1B)erythrocytes. Ten % hematocrit solution of washed red cells were incubated with equal volume of 73 mM AAPH at 37°C. *significantly different from controls, p = 0.001 for Figure 1A and P = 0.01 for Figure 1B.

deoxygenation decreased the rate of hemolysis for both sickle and control erythrocytes but the trend remained the same as in oxygenated RBCs. After 3½ hours, deoxygenated sickle erythrocytes averaged 22.8% hemolysis while control erythrocytes averaged 42.8% hemolysis (Figure 1B).

Susceptibility to Osmotic Stress-Induced Hemolysis

As with AAPH-induced hemolysis, sickled RBCs were also more resistant to osmotic stress than control RBCs at every concentration of the buffer tested (Figure 2). When the concentration of saline-phosphate buffer was decreased from 32% to 26%, hemolysis observed in sickle RBCs was increased from an average of 17.5% to 43.6%, while that of controls increased from 42.7% to 100%.

Oxidative State and Antioxidant Status

Oxidative state was evaluated by determining levels of oxidative products, MDA and conjugated dienes, in unincubated plasma and RBCs. Sickle cell patients were not significantly ($p<0.05$) different from controls in these measures of oxidative stress (Table 1). Sickle cell patients, however, had significantly lower plasma lipids and higher RBC lipids than controls.

Sickle cell patients were different from controls in certain aspects of their antioxidant status. As is shown in Table 1, the levels of GSH and plasma carotenes were significantly lower in sickle cell patients than control subjects. Sickle cell subjects had higher RBC vitamin E than controls when expressed as μg/ml packed cells but not when expressed as μg/mg lipids. The levels of whole blood ascorbic acid, plasma vitamin E and plasma retinol were not significantly different in sickle cell patients and controls.

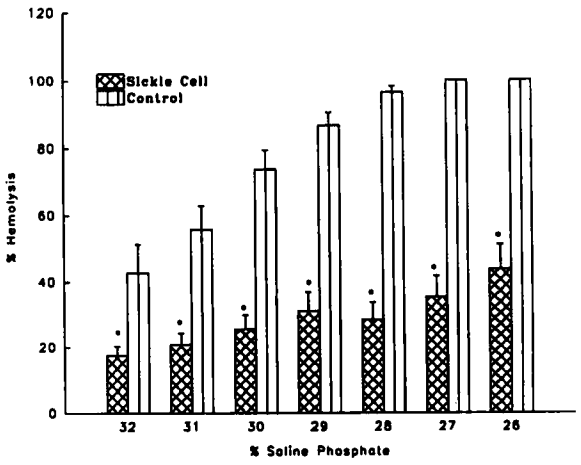

FIGURE 2 Erythrocyte hemolysis induced by osmotic stress. Washed red cells were added to hypotonic saline-phosphate buffer, pH 7.4. *Significantly different from controls ($p<0.0001$)

Effect of Nutrient Supplementation

The results described above are comparisons of sickle cell patients and controls with no distinction being made between sickle cell patients receiving nutritional supplements and those receiving no supplementation. When the same comparisons are made between controls and sickle cell patients receiving no supplements, the results were similar. In addition, there were no significant differences between sickle cell patients receiving nutritional supplements and those receiving no supplementation in any of the parameters measured (data not shown).

DISCUSSION

The mechanisms by which SCA patients develop chronic hemolysis, frequent infections, anemia and vascular occlusion are not yet clear. Sickle RBC membranes have a number of abnormalities including reduced levels of cholesterol, sialic acid and high molecular weight proteins and increased levels of lower molecular proteins.[8,20,31] Also, during the sickling process membrane phospholipids become more exposed on the outer leaflet and aminophospholipid translocase activity is reduced.[8,14] These disturbances could lead to an enhanced susceptibility to oxidative stress. Additionally, unstable sickle hemoglobin

TABLE 1 Oxidative stress and antioxidant status of sickle cell anemia patients and hospital controls

	Sickle Cell	Control
MDA (nmoles/ml plasma) n = 35	0.43 ± 0.08*	0.32 ± 0.06 (ns**)
MDA (nmoles/ml packed RBC) n = 23	5.16 ± 1.12	5.18 ± 0.65 (ns)
Conjugated Dienes (nmoles/mg lipids, plasma) n = 13	41.69 ± 8.73	38.46 ± 7.24 (ns)
Conjugated Dienes (nmoles/mg lipids, RBCs) n = 9	53.22 ± 12.4	61.33 ± 16.6(ns)
Vitamin E (μg/ml packed RBC) n = 28	2.35 ± 0.26	1.43 ± 0.31 (p = 0.0001)
Vitamin E (μg/ml plasma) n = 42	7.86 ± 0.62	8.65 ± 0.69 (ns)
Vitamin C (μg/ml whole blood) n = 19	41.41 ± 4.80	43.93 ± 4.80 (ns)
Retinol (μg/ml plasma) n = 30	0.41 ± 0.05	0.34 ± 0.05 (ns)
GSH (μMole/ml packed RBC) n = 20	1.14 ± 0.13	2.13 ± 0.08 (p < .0001)
Carotenes (μg/ml plasma) n = 21	0.43 ± 0.02	0.55 ± 0.03 (p < .01)
Total Lipids (mg/ml plasma) n = 42	3.36 ± 0.19	4.53 ± 0.26 (p < .0001)
Total Lipids (mg/ml packed RBCs) n = 32	3.51 ± 0.39	2.15 ± 0.12 (p < .0001)

*Data expressed as mean ± standard error
**Not significantly different (p>0.05)

can lead to iron decompartmentalization and increased generation of superoxide radicals, potentially magnifying oxidative stress and possibly resulting in oxidative damage to cellular lipids and proteins.[32] Increased oxidative damage has been suggested to play a role in the development of the complications associated with SCA.[8,11,15,31]

The results obtained from this study, however, showed that sickle RBCs, whether oxygenated or deoxygenated, were more resistant to AAPH-induced hemolysis than controls. AAPH, an aqueous non-membrane permeable peroxyl radical generator, is likely to attack the exterior of the RBC membrane and minimizes potential interactions with heme iron or heme derived free iron.[22] The findings suggest that the membrane abnormalities observed in the sickle RBCs do not lead to enhanced susceptibility to hemolysis induced by AAPH. Similarly, sickle RBCs were more resistant to osmotic stress than the control RBCs. It has been suggested that differences in the surface area to volume ratio of sickle RBCs may be responsible.[33] Increased resistance of sickle RBCs to hemolysis induced by osmotic stress has been reported previously and is common to several of the hemoglobinopathies.[1,34,35]

The RBCs and plasma of sickle cell patients have been shown to contain higher endogenous levels of lipid peroxidation products.[9–12,20] Also,

sickle RBCs have been shown to produce more lipid peroxidation products than normal RBCs when incubated with iron containing systems, hydrogen peroxide or other organic peroxides.[9–13] This study, using a more specific HPLC method for MDA detection, found no significant differences in unincubated sickle and control samples. Nor were sickle RBCs or plasma different from controls in levels of conjugated dienes. Also, there was no significant difference between sickle and control RBCs in MDA generation following AAPH incubation (unpublished observations). Reports concerning the susceptibility of sickle RBCs to oxidative stress are inconsistent. Schacter *et al.*,[18] for example, have found lower, rather than higher, levels of MDA in sickle RBCs than the controls. Also, augmentation of RBC antioxidant status by vitamin E and vitamin C supplementation did not change hemoglobin concentrations, RBC age or number of aplastic and vasooclusive cries of SCA patients.[21]

Several reports have attributed enhanced oxidative susceptibility of sickle RBCs to deficiencies in sickle cell antioxidant levels.[9,15–21] The present study found that sickle cell patients had lower levels of GSH and total carotenes than controls, but were not different from controls in vitamin C (ascorbic acid), retinol or plasma vitamin E. Also, sickle erythrocyte levels of vitamin E were higher than those of controls on a per volume of packed cell basis. Reports on the antioxidant status of SCA patients are inconsistent. The levels of vitamin E, for example, in the plasma of SCA patients have been reported to be both deficient[9,15,16] and no different[17] from controls. While sickle RBC levels of GSH are generally found to be lower than controls, GSH levels in sickle cell patients were not significantly different from controls.[11] The results obtained from this study suggest that, while sickle cell patients are different from controls in some aspects of their antioxidant status, these differences do not appear to be related to their susceptibility to hemolysis induced by peroxyl radicals or osmotic stress.

The reason of the increased resistance of sickle RBCs to AAPH- and osmotic stress-induced hemolysis observed in this study is not yet known. If any increased susceptibility of sickle RBCs to hemolysis is due to factors intrinsic to its membrane structure or composition, incubation with AAPH would be expected to increase sickle RBC hemolysis compared to controls. It appears that factors such as physical changes in the erythrocyte membranes brought about by reorganization of membrane lipids,[8,14] loss of deformability following sickling,[34] or sialic acid abnormalities[31,35] may play a more important role than oxidative or osmotic stress in the hemolytic events of sickle erythrocytes.

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