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MEMBRANE COMPONENTS IN THE RED CELLS OF PATIENTS WITH SICKLE CELL ANEMIA

RELATIONSHIP TO CELL AGING AND TO IRREVERSIBILITY OF SICKLING

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Summary

Cholesterol, phospholipid and sialic acid were measured in red cells from patients with sickle cell anemia to determine whether the cells had abnormal concentrations of these components and whether the amounts of these compounds differed in irreversibly sickled cells as compared to non-irreversibly sickled cells. Sickle cells had significantly higher levels of both lipids than similar populations of normal cells, however, comparisons to populations of young control cells showed that the differences were generally not significant. Sialic acid levels in sickle cells were not significantly different from normal cells. Irreversibly sickled cells had lower lipid and sialic acid concentrations than those not irreversibly sickled, but the differences were either not significant or did not occur when compared to young control cells. The studies show that the increased lipid concentrations in the membrane of sickle cells are not abnormal but are related to cell age and that the decrease in membrane components in irreversibly sickled cells is no greater than would be predicted for similarly aged populations of cells.

Introduction

Although many aspects of the red cell membrane of patients with sickle cell anemia have been extensively studied, relatively few examinations of its lipid

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components have been made. The available studies of membrane lipid have shown increased concentrations in sickle cells [1,2], no significant phospholipid loss in the formation of irreversibly sickled cells [3], and no significant decrease of phospholipid or cholesterol in membranes of irreversibly sickled cells [4]. The extent to which these findings interrelate or the degree to which other membrane components, such as sialic acid, are associated with membrane abnormalities is not known although lipid abnormalities may be associated with changes in cell shape [5] and sialic acid is closely related to the electrical charge and viscosity of the red cells [6]. For these reasons and since earlier studies of sickle cells have not fully considered that membrane lipid may be a function of cell aging [7—9], the present study was done. In this study phospholipid, cholesterol and sialic acid were measured at various levels of centrifuged columns of sickle cells which included fractions which were rich in irreversibly sickled cells as well as fractions of non-irreversibly sickled cells. The results were related to corresponding control populations of young cells and normal cells. Age-related cell enzyme assays were made to determine the relationship between the concentrations of the membrane components and cell aging. Osmotic fragility of the various red cell fractions was measured to clarify the extent to which the irreversibly sickled cells might affect the membrane measurements in each fraction since the irreversibly sickled cell fractions were not completely pure.

Methods

Blood samples were obtained from normal individuals, from patients with sickle cell anemia and from patients with reticulocytosis associated with various types of anemia. The patients with sickle cell anemia had typical clinical findings of sickle cell anemia. None were in vaso-occulsive crises. Hemoglobin electrophoresis on these patients showed only S, A_2 and F hemoglobin. None had been transfused in the preceding four months. The types of anemia in the patients with reticulocytosis were drug-induced hemolytic anemia, acquired non-spherocytic hemolytic anemia (non-gamma type), acute lead poisoning, paroxysmal nocturnal hemoglobinuria and megaloblastic anemia under treatment. Comparisons of lipid concentrations to these reticulocyte-rich populations of cells seemed appropriate since none of these disorders had cell lipid abnormalities or have been shown to have persistent and significant cell lipid changes [5], while they all had reticulocytosis. All studies were performed on venous blood using ethylenediaminetetraacetate (1 mg/1 ml blood) or heparin (15 IU/1 ml blood) as the anticoagulant. No differences between the two anticoagulants were observed.

Sample separation was obtained by the method of Bertles [10] with minor modifications. Venous blood was oxygenated by equilibrating it with a 95% O_2/5% CO_2 gas mixture for 10—15 min. The hematocrit was adjusted to approximately 80%, the sample placed in cellulose acetate tubes 1.25 cm × 5.0 cm with total volume approximately 5.5 ml and centrifuged at 40 000 rev./min (175 000 × g) in a Spinco SW 50L swinging bucket rotor for 60 min at 20° C. The buffy coats were removed, and cell samples were obtained by slicing each tube into a top, middle and bottom layer with a tube cutter. Each level contained approximately 1.6 ml of cells. Meticulous care was taken in the

use of the tube cutter to minimize turbulence and to avoid mixing of the cells of different levels. The cells were then suspended in autologous plasma. Aliquots of top, middle and bottom layers were analysed.

Reticulocyte counts were made using standard methods. Irreversibly sickled cells were obtained and evaluated as described by earlier methods after equilibration of cells with 95% O_2/5% CO_2 [10]. The range of the mean percentage of irreversibly sickled cells in patients was 8—38%. Lipid determinations were obtained by methods previously described [11]. Red cell enzymes glutamic oxaloacetic transaminase, glucose-6-phosphate dehydrogenase were assayed by standard methods [12]. Sialic acid content of cells was measured by the techniques described by Tishkoff [13]. Red cell osmotic fragility was determined with a Fragiligraph (Model D 2, Elron Electronic Industries, Israel Ltd.) on freshly prepared samples of red cells at 21 ± 0.5° C. All specimens were measured with a normal blood sample and with a dialysis membrane which had been standardized.

Results

Lipid and sialic acid measurements

Comparisons between comparable layers of sickle and normal cells showed sickle cells to have higher phospholipid concentrations in the top ($P < 0.05$) and middle layers ($P < 0.05$) and higher cholesterol concentrations in all layers (top $P < 0.01$; middle $P < 0.05$; bottom $P < 0.05$) than those of normal cells (Table I, Fig. 1). Comparisons between the sickle cells and the reticulocyte-rich cells showed the reticulocyte-rich cells to have a higher concentration of phospholipid in the middle layer ($P < 0.01$) as compared to a similar layer of sickle cells while cholesterol and sialic acid levels were not consistently or significantly different from those obtained in sickle cells (Table I, Fig. 1). In comparisons between reticulocyte-rich cells and normal cells, all layers of reticulocyte-rich cells had increased lipid concentrations (phospholipid: top $P < 0.05$, middle $P < 0.01$, bottom $P < 0.05$; cholesterol: top $P < 0.01$, middle $P < 0.01$, bottom $P < 0.05$) (Table I, Fig. 1). Sialic acid levels were not significantly different between these two types of cells.

Phospholipid, cholesterol and sialic acid concentrations decreased with progression down the columns of the sickle cells, normal cells and reticulocyte-rich cells with the exception of sialic acid levels in normal cells (Table I, Fig. 1). Few statistically significant differences were observed (sickle cells: phospholipid, middle vs. bottom $P < 0.05$; cholesterol, top vs. middle $P < 0.01$; reticulocyte rich: phospholipid, middle vs. bottom $P < 0.01$). The phospholipid : cholesterol ratios varied between 2.4 and 3.0 in the different layers of all columns. None of the differences were significant.

Enzyme activity in the various populations of cells

Sickle cells and reticulocyte-rich cells had higher glutamic oxaloacetic transaminase ($P < 0.01$) and glucose-6-phosphate dehydrogenase activity ($P < 0.01$) in all layers compared to similar layers of normal cells (Table II, Fig. 2) while glucose-6-phosphate dehydrogenase activity was greater in the bottom layer of reticulocyte-rich cells as compared to the bottom layer of sickle cells ($P <$

TABLE I

CONCENTRATIONS OF STRUCTURAL MEMBRANE COMPONENTS IN SICKLE RED CELLS AND CONTROL CELLS AT VARIOUS LEVELS OF CENTRIFUGED CELL COLUMNS

Ten specimens from ten different individuals were examined in each group. Phospholipid and cholesterol data are in mg/cell ($\times 10^{-12}$); sialic acid in μg/cell ($\times 10^{-9}$). Data are mean ± S.E.

	Sickle cells			Normal control			Reticulocyte-rich control		
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
Phospholipid	336.7 ± 15.5	311.6 ± 10.1	279.2 ± 12.0	283.4 ± 14.0	273.9 ± 6.6	253.0 ± 13.0	379.2 ± 17.6	357.6 ± 12.3	297.2 ± 16.5
Cholesterol	135.9 ± 4.9	119.9 ± 2.8	116.9 ± 3.2	112.1 ± 4.0	108.5 ± 3.6	107.1 ± 3.5	128.9 ± 6.2	125.6 ± 5.2	118.8 ± 3.5
Sialic acid	18.3 ± 0.65	17.7 ± 0.80	17.3 ± 0.93	16.5 ± 0.69	17.8 ± 0.67	17.5 ± 0.63	19.1 ± 3.1	18.0 ± 3.1	15.0 ± 2.4
Reticulocytes (%)	30.5 ± 2.4	8.4 ± 1.6	3.2 ± 0.52	1.6 ± 0.05	0.21 ± 0.05	0.17 ± 0.07	29.3 ± 6.0	10.8 ± 2.5	3.8 ± 0.81
Irreversibly sickled cells (%)	1	4	52						

TABLE II

ENZYME ACTIVITY IN SICKLE RED CELLS AND CONTROL CELLS AT VARIOUS LEVELS OF CENTRIFUGED COLUMNS

Ten specimens from ten different individuals were examined in each group. Data are in IU/10^{10} RBC, and are mean ± S.E.

	Sickle cells			Normal control			Reticulocyte-rich control		
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
Glutamic oxaloacetic transaminase	1.93 ± 0.17	1.46 ± 0.11	1.13 ± 0.10	0.78 ± 0.08	0.50 ± 0.04	0.44 ± 0.03	2.91 ± 0.56	1.89 ± 0.28	1.36 ± 0.25
Glucose-6-phosphate dehydrogenase	2.68 ± 0.25	1.96 ± 0.30	1.66 ± 0.20	1.40 ± 0.14	0.93 ± 0.09	0.88 ± 0.08	2.96 ± 0.37	2.73 ± 0.49	2.55 ± 0.36
Reticulocytes (%)	30.5 ± 2.4	8.4 ± 1.6	3.2 ± 0.52	1.6 ± 0.25	0.21 ± 0.05	0.17 ± 0.07	29.3 ± 6.0	10.8 ± 2.5	3.8 ± 0.81
Irreversibly sickled cells (%)	1	4	52						

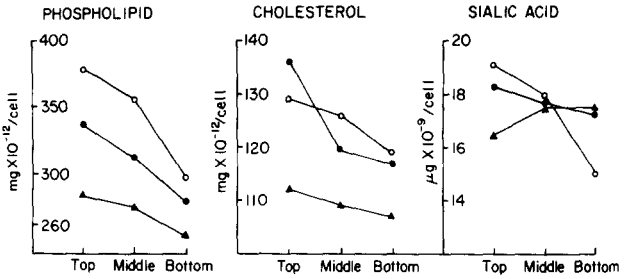

Fig. 1. Concentration of phospholipid, cholesterol and sialic acid in sickle (●———●), normal (▲———▲), and reticulocyte-rich (○———○) red cells. Cells were obtained from the top, middle and bottom layers of centrifuged cell columns.

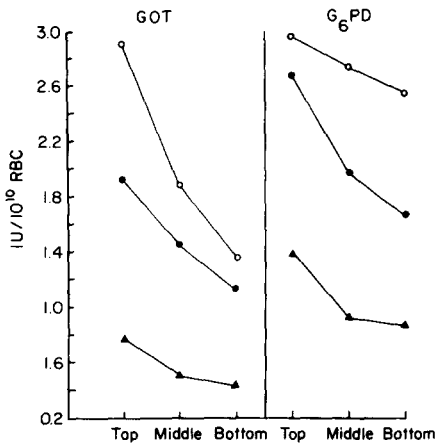

Fig. 2. Enzyme activity in sickle (●———●), normal (▲———▲) and reticulocyte-rich (○———○) red cells obtained from top, middle and bottom layers of centrifuged columns. GOT, glutamic oxaloacetic acid; G6PD, glucose-6-phosphate dehydrogenase.

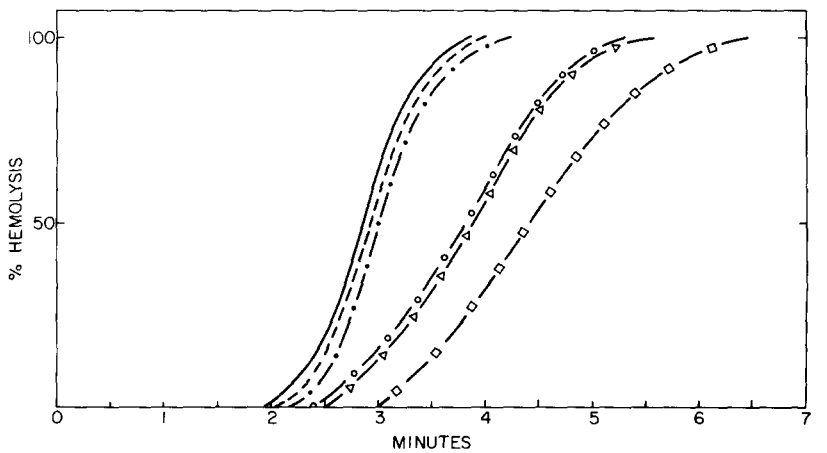

Fig. 3. Red cell osmotic fragility in top, middle and bottom layers of normal and sickle columns of red cells. Each curve represents a mean value. Ten normal individuals and 12 sickle patients were evaluated. Top normal (●———●), middle normal (- - - - -), bottom normal (———); Top sickle (△———△), middle sickle (○———○), bottom sickle (□———□).

0.05). Activities of both enzymes decreased with progression down the columns of sickle cells, normal cells and reticulocyte-rich cells (sickle cells: glucose-6-phosphate dehydroganse, top vs. middle $P < 0.05$, middle vs. bottom $P < 0.05$; normal cells: glutamic oxaloacetic transaminase, top vs. middle $P < 0.01$; glucose-6-phosphate dehydrogenase, top vs. middle $P < 0.01$) (Table II, Fig. 2).

Comparisons between the concentrations of phospholipid and cholesterol and the activities of glutamic oxaloacetic transaminase and glucose-6-phosphate dehydrogenase of the different types of cells at similar levels of the cell columns shows high correlations ($r = 0.8$).

Osmotic fragility of the various populations of cells

Osmotic fragility curves of the various layers of sickle cells showed only single populations of cells. Although normal cells became less resistant to osmotic lysis with progression down cell columns, the bottom layer of sickle cells was more resistant to osmotic lysis (Fig. 3).

Discussion

The present study shows that sickle cells have higher lipid levels than normal cells, however, comparisons to young control cells and to age-related enzyme activities demonstrate that the differences are no greater than would be predicted for the age of the cell. The lipid levels of sickle cells were similar to those of similarly layered reticulocyte-rich cells and significantly higher than layered normal cells. The age-related enzymes, glutamic oxaloacetic transaminase and glucose-6-phosphate dehydrogenase, also showed changes in the layered sickle cells which were similar to the lipid alterations and were compatible with aging differences between the cell layers [14,15]. The previous studies on sickle cell lipids have described significantly increased lipid levels [1,2], however, the results were compared to normal cells without full consideration that red cell lipid levels appear to be a function of aging [7—9]. The present results confirm previous studies of lipid changes in normal aging cells [7—9] and would require consideration when membranes from sickle cells are evaluated.

In this study, the irreversibly sickled cell populations had lower phospholipid and cholesterol levels than the non-irreversibly sickled cell populations. Comparable layers of young control cells as well as the age-related enzymes, glutamic oxaloacetic transaminase and glucose-6-phosphate dehydrogenase, however, showed similar decreases while the phospholipid : cholesterol ratio was similar in both types of cells. Thus, although the irreversibly sickled cell fractions contain less phospholipid and cholesterol than the non-irreversibly sickled cell fractions, the differences in lipid levels between them do not show consistent statistical changes and are comparable to the differences obtained in control young cells and in age-related enzyme activities.

The possibility that the lipid loss in the irreversibly sickled cell fraction was underestimated was considered since these fractions usally contained 50% rather than 100% irreversibly sickled cells. This would seem unlikely, however, since the lipid loss as calculated by extrapolating the results to 100% irreversibly sickled cells did not differ significantly from results obtained in similar populations of reticulocyte-rich control cells. Further evidence that significant

number of irreversibly sickled cells were present was obtained from osmotic fragility studies of the cells. Irreversibly sickled cells should be less fragile than non-irreversibly sickled cells because of decreased cation content [4,16]. As shown in our study, the irreversibly sickled cell fraction had the predictable fragility changes for irreversibly sickled cells and are compatible with a high concentration of the cells. The abnormal fragility of native irreversibly sickled cells has not previously been described although it has been noted (Bertles, J.F., personal communication) and observed in calcium-induced 'irreversibly sickled cells' [17]. The findings reenforce the limited previous findings that irreversibly sickled cells have not lost excessive membrane [3,4]. The results do not negate that fragmentation with subsequent membrane loss does not occur [18], but suggest that it is not a major factor in the formation of irreversibly sickled cells. Reorganization of the lipids in the membrane of irreversibly sickled cells may occur without lipid loss [19], or parts of membrane may be lost rather than complete pieces.

Changes in sialic acid have not been associated with abnormalities in cell shape, however, its importance in the electrical charge and viscosity [6] of the cell as well as its association with a shortened cell life span [20] could be a significant factor in explaining certain properties of sickle cells and the irreversibly sickled cells. Although the present results shows that sialic acid concentrations are lower in irreversibly sickled cells as compared to non-irreversibly sickled cells, the differences are not statistically significant nor are other differences between sialic acid content of sickle cells and control cells constant. The sialic acid results confirm the lipid measurements showing that excessive membrane is not lost in the formation of irreversibly sickled cells.

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