Lipid Synthesis in Human Erythroid Cells: The Effect of Sickling

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Human reticulocytes are capable of synthesizing membrane lipids from ^{14}C-glycerol de novo. In both sickle and nonsickle reticulocytes the majority of ^{14}C-glycerol was incorporated into phospholipids, primarily phosphatidylserine and phosphatidylcholine. Incorporation into sphingomyelin was minimal. The most abundant neutral lipid synthesized was triglyceride. In the absence of sickling, the rate of lipid synthesis in sickle reticulocytes was similar to that of nonsickle reticulocytes. With the induction of sickling under anoxic conditions sickle reticulocytes showed a prompt increase in the rate of lipid synthesis to an average of 69% above control values, while nonsickle reticulocytes under similar conditions decreased the rate of lipid synthesis. An increase in the rate of membrane lipid synthesis is associated in the mammalian erythroid cell with cell membrane damage. The findings further confirm that lesions of the erythroid cell membrane in sickle cell anemia are secondary to the sickling process itself.

PREVIOUS STUDIES in this laboratory have shown that damage to mammalian reticulocyte membranes is associated with an increased rate of lipid synthesis.[1] These two phenomena are associated in the laboratory[2] as well as in clinical conditions.[2,3] Sickling is known to cause increased cation flux across the erythrocyte membrane,[4] accumulation of membrane calcium,[5] and loss of membrane mass.[6] The eventual result of these alterations is permanent membrane damage and the irreversibly sickled cell.[7,8] If physical damage to the cell membrane is a secondary event during sickling, it would be expected also to be associated with an increased rate of lipid synthesis.

These studies have been designed to investigate synthesis de novo of lipids in the human erythroid cell and to determine how damage to the cell membrane affects this process. In human reticulocytes, as in other mammalian reticulocytes,[1] damage to the cell membrane is associated with an increase in the rate of lipid synthesis de novo. When sickling is induced there is also an associated increased rate of lipid synthesis. Since the rate of lipid synthesis in sickle reticulocytes in the absence of sickling is *not* increased above that of other types of reticulocytes, these observations confirm that lesions of the erythroid cell membrane in sickle cell anemia are secondary to the sickling process itself.

MATERIALS AND METHODS

The methods have been described in detail in earlier publications.[1,9] Blood with a reticulocyte count between 5% and 13% was obtained from patients with sickle cell anemia or autoimmune hemolytic anemias, or from normal human placentas at parturition. All blood was collected in heparinized tubes and unless otherwise specified all procedures were performed at 0°–4°C.

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Conditions of incubation. The incubation mixture, usually 8 ml in total, contained equal amounts of washed packed cells and Krebs-Ringer buffer containing glucose, iron, and amino acids[10] and 10^{-4} *M* calcium chloride;[11] 1-5 μCi of ^{14}C-glycerol was added and the incubations were carried out as described with agitation at 37°C. In experiments in which incubations were carried out under anoxic conditions, an atmosphere of 100% nitrogen was supplied either by continuous flow or by exposure for 5 min prior to sealing the tubes. Under these conditions, the pO_2 was consistently reduced in 15 min to less than 20 mm mercury, and there was at least a threefold increase in the number of sickled forms on peripheral smears of formalinized preparations. No increase in sickled forms occurred in sickle cells incubated under room air. Vincristine was used in a final concentration of 1.5×10^{-4} *M*, and puromycin was added to a final concentration of 10^{-3} *M*. Hydrogen peroxide was added as a 30% solution to bring the cell suspension to a final concentration of 4%, and the cells were washed with 5% thymol after incubation.[3] These concentrations of agents did not result in spontaneous hemolysis of the cells greater than that observed in control.

Incubations were terminated by the addition of 2 volumes of ice-cold phosphate-buffered saline, and the cells were washed twice in the same solution. The washed cells were lysed with 4 volumes of 20 mOsm phosphate buffer, pH 7.4, and the cell membranes separated by centrifugation at 43,500 *g* for 10 min. The separated membranes were washed three times with 20 mOsm buffer.

Extraction and analysis of membrane lipids. The washed membranes, suspended in 2 ml of 20 mOsm phosphate buffer were extracted with 5 ml each of methanol and chloroform.[1] The membrane lipids were analyzed by thin layer chromatography on silica gel G, silicic acid column chromatography, and partial alkaline hydrolysis.[1,9] The accuracy of these methods has been documented.[1,9]

Analytic methods. Hemocytometry, enumeration of reticulocytes, and hematocrit determinations were done by standard methods.[12] The percentage of sickle cells was determined by anerobically transferring 20 microliters of cell suspension into 2 ml of 10% formalin in buffered normal saline. After 10 min the suspension was centrifuged at 900 *g* for 10 min, and the cell button was resuspended in 0.1 ml of the buffered formalin. The smears were stained with Wright's stain and examined under oil immersion microscopy. Radioactivity was determined in a liquid scintillation counter.[10]

Materials. All chemicals were reagent grade. Labeled precursors were obtained from New England Nuclear, Boston, Mass. Precoated thin layer chromatographic plates were obtained from Brinkman Instrument, Inc. Vincristine and vinblastine were standard commercial pharmaceuticals. Puromycin dihydrochloride was obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

RESULTS

Lipid Synthesis in Human Reticulocytes

Human reticulocytes are capable of synthesizing lipids de novo from ^{14}C-glycerol (Fig. 1). An average of 57% (range, 55%-75%) of ^{14}C-glycerol is incorporated into phospholipids, while an average of 43% (range, 21%-45%) of the precursor is incorporated into neutral lipids (Table 1). Analysis of the newly synthesized phospholipids by partial alkaline hydrolysis shows that more than 90% of the precursor ^{14}C-glycerol is incorporated into the 3-carbon glycerol backbone, which can only represent de novo phospholipid synthesis,[13] and less than 10% is in the fatty acid side chains. Thirty-seven per cent of the incorporated precursor appears in phosphatidylserine and phosphatidylcholine, and 17% in phosphatidylethanolamine. Incorporation into sphingomyelin is minimal. Incorporation of ^{14}C-glycerol into neutral lipids by human reticulocytes is mainly into triglycerides (33% of total incorporation), while cholesterol (7%) and diglycerides (3%) are synthesized to a lesser extent (Table 1).

In order to determine if human reticulocytes increase the rate of lipid synthesis when the cell membrane is damaged, reticulocytes from patients with

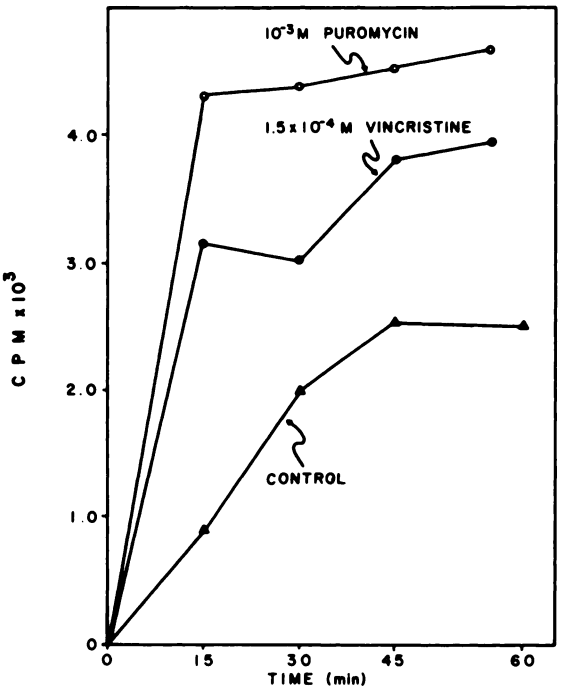

Fig. 1. The incorporation of ^{14}C-glycerol into human reticulocyte lipids. Washed reticulocyte-rich blood was incubated as described in Methods at 37°C for 1 hr under room air. At intervals during the incubation the washed membranes from the cells were examined for incorporation of glycerol into the isolated lipids. Both puromycin and vincristine increased the rate of incorporation of ^{14}C-glycerol into reticulocyte lipids.

hemolytic anemia were incubated in the presence of 1.5×10^{-4} *M* vincristine or 10^{-3} *M* puromycin, agents which cause endocytosis of the erythrocyte membrane and cell vacuolization.[14,15] Exposure to either agent produces a prompt and substantial stimulation in the rate of ^{14}C-glycerol incorporation into total cell lipids (Fig. 1 and Table 2). As in rabbit reticulocytes,[1] this increase in the rate of lipid synthesis is accompanied by a change in the proportion of precursor incorporated into individual lipid classes. For example, in reticulocytes from patients with hemolytic anemia, exposure to vincristine causes a 92% increase in neutral lipid synthesis and only a 24% increase in phospholipid synthesis. Exposure to puromycin has an opposite effect, increasing phospho-

Table 1. Incorporation of ^{14}C-Glycerol Into Human Reticulocyte Lipids

	Total Lipid Synthesized (%)	cpm ± SE*
Neutral lipids		
Triglyceride	33	10,600 ± 1723
Diglyceride	3	800 ± 141
Cholesterol	7	2,200 ± 638
Total	43	13,600 ± 2422
Phospholipids		
Phosphatidyl-ethanolamine	17	5,360 ± 2700
Phosphatidyl-choline and serine	37	11,600 ± 3500
Sphingomyelin	3	890 ± 420
Total	57	17,900 ± 6700

*Counts per minute incorporated into lipids in a 50% cell suspension with approximately a 7% reticulocyte count. Figures are averages of seven separate experiments.

Table 2. Effect of Membrane Damage on Lipid Synthesis in Human Reticulocytes in Hemolytic Anemia and Sickle Cell Anemia

	Total Lipids		Neutral Lipids		Phospholipids	
	cpm*	Increase (%)	cpm	Increase (%)	cpm	Increase (%)
Hemolytic anemia						
Control	23.6 ± 7.8	—	8.2 ± 0.8	—	15.3 ± 6.5	—
Vincristine						
1.5×10^{-4} M	34.9 ± 11.6	+48	15.8 ± 0.1	+92	19.0 ± 11.5	+24
Puromycin						
10^{-3} M	37.8 ± 9.2	+60	10.5 ± 3.9	+28	27.3 ± 11.1	+77
Sickle cell anemia						
Control	22.3 ± 2.4	—	9.7 ± 0.2	—	12.6 ± 1.4	—
Vincristine						
1.5×10^{-4} M	36.2 ± 7.6	+63	15.1 ± 3.8	+56	21.7 ± 4.2	+72
Puromycin						
10^{-3} M	43.9 ± 8.9	+97	18.1 ± 5.2	+86	25.9 ± 8.2	+106

*Counts per minute $\times 10^3$ ± SE. A 50% suspension of reticulocyte-rich blood was incubated for 1 hr at 37°C with either 10^{-3} M puromycin, 1.5×10^{-4} M vincristine, or an equal volume of normal saline for controls. Resultant synthesis of lipids was analyzed as described in Methods. Figures represent averages of at least four separate studies. The differences in stimulation between sickle and hemolytic anemia cells are not significant.

lipid synthesis 77% and neutral lipid synthesis only 28%. Incubation of cells with 3.6×10^{-3} *M* butanol, 1.5×10^{-4} *M* vinblastine, or 4% hydrogen peroxide also results in a stimulation of the rate of total lipid synthesis by 122%, 169%, and 414%, respectively.

Lipid Synthesis in Sickle Cell Anemia Reticulocytes

In order to determine if the characteristics of lipid synthesis in sickle cell anemia reticulocytes differed from those of nonsickle reticulocytes, the rate of lipid synthesis in these cells was compared to that of human fetal reticulocytes and reticulocytes from patients with hemolytic anemia. The effects of different degrees of cell maturity in the blood was minimized by selecting only samples with a reticulocytosis between 5% and 15%. There was a general relationship between reticulocyte count in a sample and the rate of lipid synthesis. However, the average rate of lipid synthesis in ten sickle cell anemia samples did not differ from that of ten cord blood samples and ten hemolytic anemia samples with comparable reticulocyte counts. Thus, under room air the baseline rate of lipid synthesis in sickle cell anemia reticulocytes was, per unit of cells, not higher than that of the other types of cells studied. Both sickle reticulocytes and nonsickle reticulocytes were able to increase further the rate of lipid synthesis when exposed to agents which damage the cell membrane. The rate of lipid synthesis in sickle reticulocytes exposed to 1.5×10^{-5} *M* vincristine or 10^{-3} *M* puromycin was increased by an average of 63% and 97%, respectively (Table 2). These findings indicate that the pathways for lipid synthesis in sickle reticulocytes were not functioning at maximum capacity when sickling did not occur.

A direct approach to the question of whether or not sickling was associated with increased lipid synthesis was obtained by comparing the rate of lipid syn-

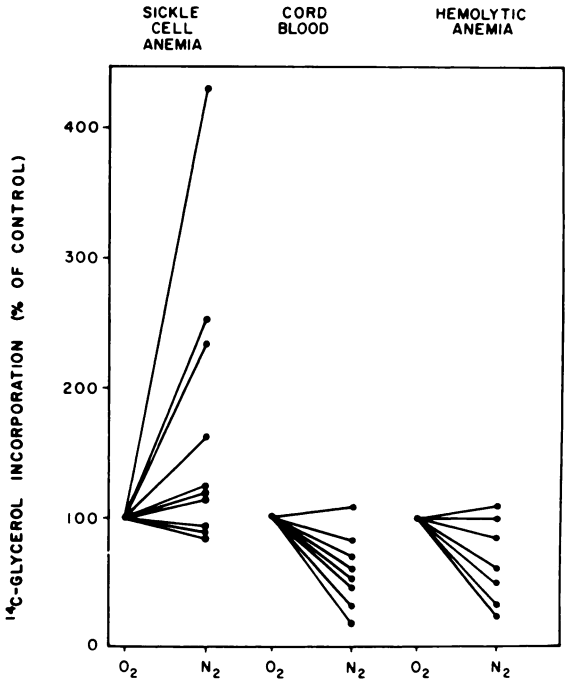

Fig. 2. The effect of anoxia on lipid synthesis in human reticulocytes. Blood from patients with either sickle cell anemia or hemolytic anemia, or from umbilical cords was incubated as described in Methods under an atmosphere of room air or under an atmosphere of 100% nitrogen. Incorporation of ^{14}C-glycerol into total reticulocyte lipids was determined after incubation at 37°C for 1 hr.

thesis in sickle reticulocytes incubated under air with that of those incubated under hypoxic conditions. When incubated under anoxic conditions for 1 hr at 37°C, sickle forms increased at least threefold. Although there was variation in the degree of response, in seven of ten studies sickling was associated with an increase above that of control values in incorporation of glycerol into total cell lipids. The average increase was 69% (Fig. 2). In contrast, glycerol incorporation in fetal cells and in cells from hemolytic anemia patients under anoxic conditions fell in all cases, to an average of 62% and 61% of control values, respectively.

DISCUSSION

This study clarifies the understanding of erythrocyte membrane lesions in sickle cell anemia. In the absence of sickling, the rate of lipid synthesis in sickle reticulocytes is both quantitatively and qualitatively similar to that of nonsickle reticulocytes. Recognizing that reticulocytes are a heterogeneous population with respect to age, this statement can be made because it is unlikely that, in this large number of samples with comparable reticulocytoses, there are significant differences in cell maturity and metabolic capabilities. Nevertheless, we cannot exclude the possibility that differences in cell maturity might affect the results. There is, however, additional evidence that in the oxygenated state sickle and nonsickle reticulocytes are similar with respect to the rate of lipid synthesis. Both types of cells show a further and similar increase in lipid synthesis when the membrane is damaged by endocytotic agents. Since in the absence of sickling the pathways for lipid synthesis in sickle reticulocytes are not

functioning at a rate greater than that of nonsickle reticulocytes, and since membrane damage is associated with an increased rate of lipid synthesis, the data suggest that membrane integrity in the sickle reticulocyte is normal until sickling occurs. This conclusion is consistent with the fact that abnormal cation fluxes in sickle erythrocyte membranes do not occur until sickling is induced,[4] despite the increased binding of hemoglobin S to the cell membrane.[16]

The prompt increase in the rate of erythroid cell lipid synthesis when sickling is induced is a response characteristic of conditions in which the cell membrane is damaged[1] and adds further confirmation that it is the sickling process itself which damages the erythroid cell membrane. The recent report that normal erythrocyte membranes, when filled with hemoglobin S, will sickle under anoxic conditions[17] supports this interpretation. That the response of sickle reticulocytes in increasing lipid synthesis during incubation in the absence of oxygen is related to the sickling phenomenon and is not a nonspecific effect of hypoxia is confirmed by the finding that other types of human reticulocytes do not increase lipid synthesis under identical conditions.

This study also indicates that there are some differences in lipid synthesis in human reticulocytes as compared to rabbit reticulocytes.[1,9] Human reticulocytes incorporate approximately equal proportions of ^{14}C-glycerol into phospholipids and neutral lipids, while in rabbit reticulocytes 70% of the precursor glycerol is found in phospholipids.[9] Human reticulocytes also do not utilize glycerol to any great extent in the synthesis of cholesterol, most of the precursor being incorporated into triglycerides. These differences may reflect different metabolic requirements for the synthesis of lipids in the erythroid cells of the two species, despite the relatively minor differences in composition of human and rabbit erythroid cell membranes,[18,19] or may be due to differences in degree of cell maturity between the samples. The response to endocytotic agents is not precisely the same in human and rabbit reticulocytes. The selective effect of vincristine on neutral lipid synthesis, and puromycin on phospholipid synthesis, in rabbit cells[1] is much less defined in human cells. Although it has been postulated that these responses reflect the precise type of damage sustained by the membrane,[1] the significance of these selective effects remains unknown.

Since sickling has been demonstrated in reticulocytes[20] as well as mature cells, it is not surprising that the sickling process, and the resultant membrane damage, is accompanied by a response mechanism associated with other types of membrane damage. It is possible that the ability of human reticulocytes to synthesize cell membrane lipids in a selective fashion in response to membrane damage may represent a protective mechanism for these cells. In support of this hypothesis is the finding that vitamin E-deficient rat reticulocytes, which are normally resistant to peroxide hemolysis, do hemolyze when pathways of lipid synthesis are inhibited.[2,9] Evidence has been presented that cyanate, an agent used therapeutically to modify sickle erythrocytes, inhibits pathways of lipid synthesis in reticulocytes[21] and thus may impair a protective mechanism of the cell. The findings indicate that study of membrane function is of importance in the assessment of protein-modifying agents being considered for therapeutic use.

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