The Role of Red Cell Energy Metabolism in the Generation of Irreversibly Sickled Cells In Vitro

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An acquired membrane defect is believed to be responsible for the maintenance of the sickled shape in oxygenated irreversibly sickled cells (ISC), because the hemoglobin S in these cells is not in the aggregated, "sickled" state. In the present study, it is demonstrated that the acquisition of the membrane defect in vitro depends on cellular metabolism. Only if cellular ATP is almost completely depleted while the cells are sickled, do they become unable to resume the biconcave disk shape upon reoxygenation. If calcium is omitted from the incubation buffer, ISCs are not generated despite metabolic depletion. This suggests an action of ATP mediated through calcium metabolism similar to that which prevents membrane stiffening in normal red cells. No ISCs were produced by repeated sickling and unsickling. Thus, a membrane alteration occurring as a consequence of metabolic depletion seems to be a more important factor in the generation of ISC than sickling-unsickling induced fragmentation.

A VARIABLE PROPORTION (from 2% 30%) of erythrocytes in the blood of patients with sickle cell anemia are irreversibly sickled cells (ISC). These cells retain an elongated and pointed shape even after the blood is equilibrated with 100% oxygen. Despite the sickled shape of ISC, the hemoglobin in these cells is not in the aggregated, "sickled" state, since the microfilaments characteristic of deoxy-Hb S aggregates cannot be detected in electron micrographs of oxygenated ISC.[1] This apparent paradox has led to the suggestion that ISC must be generated by an acquired membrane defect which renders some of the cells incapable of returning to the biconcave shape following deoxygenation-induced sickling.[2,3,4]

One theory offered to explain this acquired membrane abnormality is based upon the known tendency of sickle cells to exhibit fragmentation during the unsickling process.[5,6] It is suggested that sickle cells undergo membrane loss during their life span in vivo and when they are juxtaposed to littoral phagocytes. When the loss of membrane reaches a critical point, the cell is supposed to be unable to change its shape, and to become fixed in the sickled form. This concept of membrane shortage as the cause of irreversible sickling is not, how-

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ever, consistent with the fact that reduction of the ratio of surface area to volume in all red cells including sickle cells leads to the development of spherocytes rather than sickled forms.

The studies of Shen and Castle[7] and their co-workers suggest a metabolic basis for the development of ISC. They showed that ISC could be produced by prolonged incubation of sickle cells under nitrogen, and they further noted that oxygenated sickle cells can be rendered irreversibly unsicklable if they are subjected to prolonged incubation without added glucose under oxygen. In support of the latter observation is the well-known clinical fact that the sodium metabisulfite screening test may be falsely negative when performed on sickle cells that have been stored for prolonged periods without the addition of glucose.

Since present evidence indicates that membrane deformability as well as membrane consistency is dependent upon the maintenance of a critical level of intracellular ATP,[8] we have compared the effects of red cell ATP depletion with membrane phospholipid depletion on the development of ISC in vitro. The results indicate that the level of ATP is far more relevant to the production of ISC than is the maintenance of red cell membrane phospholipid.

MATERIALS AND METHODS

Heparinized blood specimens were drawn during routine examinations of homozygous sickle cell anemia patients. None of the patients had any form of sickle cell anemia crisis when the samples were obtained. After centrifugation of the blood, cells and plasma were separated, and the erythrocytes were washed three times in Krebs-Henseleit buffer, (KHB) with the following composition:

	meq/liter
NaCl	123.2
KCl	5.0
KH_2PO_4	1.24
$MgSO_4 \times 7\ H_2O$	1.24
$NaHCO_3$	52.0
$CaCl_2 \times 2\ H_2O$	0.23

Adjust to 290 ± 10 mOs/liter ("Osmette," Precision Systems, Framingham, Mass.)

They were then resuspended to a hematocrit of 25% in their own plasma, which had been diluted 1 : 1 with Krebs-Henseleit buffer, or in KHB alone. Two hundred units of penicillin and 0.2 mg of streptomycin were added per ml of cell suspension. The mixture was then sterilely transferred to 50-ml Erlenmeyer flasks. Additives were first dissolved in water at 100 times their final concentration so that the desired concentration was achieved by addition of 0.01 volumes of the additive to 1 volume of red cell suspension. After removal of an aliquot for initial determinations, the flasks were closed with rubber stoppers. A continuous flow of N_2 and 5% CO_2 at 100 ml/min was then maintained through 18-gauge hypodermic needles which perforated the stoppers. Incubations were continued for 24 hr at 37°C at 80 oscillations/min. The samples were then reoxygenated for 75 ± 15 min under a stream of O_2 and 5% CO_2 at 300 ml/min. After the first 30 min of reoxygenation, the PO_2 was measured (PO_2 gas analyzer, Model 113, Instrumentation Laboratory Corp., Lexington, Mass.) and in all cases found to be higher than 300 torr. Cells that were still sickled after this this treatment were defined as ISC. pH variations after the 24-hr incubation ranged from 7.2 to 7.7 with a slightly lower range (7.2 7.6) when glucose or adenosine were present, as opposed to 7.55 7.7 in incubations without these additives.* Hemolysis was less than 1% as determined by spectrophotometric measurements of free Hb in the incubation medium.

*Small losses of CO_2 from some of the samples during pH determination may have occurred and may account for some of the higher pH values. Otherwise, the range of pH is interpreted as being due to differences in lactate production.

Table 1. The Effect of Prolonged Incubation Under N_2 + 5% CO_2 on the Percentage of ISC

Patient No.	% ISC Before Incubation	% ISC After Incubation	% ISC Produced
1	10.0	45.0	39
2	16.8	74.0	69
3	8.0	53.2	49
4	21.5	63.7	53
5	26.0	55.0	39
6	28.3	74.5	69
7	6.0	51.0	48
8	25.9	64.7	52

Cells incubated for 24 hr at 37°C in patients' sera diluted 1 : 1 with Krebs Henseleit buffer.

$$\% \text{ ISC produced} = \frac{\text{ISC after incubation} - \text{ISC before incubation}}{100 - \text{ISC before incubation}} \times 100.$$

Red cell phospholipid extraction and phosphorus determination were carried out in duplicate according to previously published methods.[9,10] ATP was measured with the PGK method as described by Adam.[11] Hematocrits were determined in duplicate in a microhematocrit centrifuge. The relative proportions of reversibly or irreversibly sickled cells and nonsickled cells were established by differentiating 1000 or more cells on wet preparations after anaerobic fixation in 10% formaldehyde in saline. The criteria established by Bertles et al.[12] ("elongated, sickled, or double pointed shapes") were used; the error in repeated counts was less than ± 5% as noted by these workers.

RESULTS

The percentage of ISC in fresh blood of eight sickle cell anemia patients ranged from 6% to 28%. In every case, this proportion could be increased by a 24-hr incubation under nitrogen, after which 45%–74% of the cells were irreversibly sickled (Table 1). Thus, between 39% and 69% of the non-ISC hemoglobin-S cells had been converted to ISC by this procedure. Photographs of these cells (Fig. 1A) demonstrate that they fulfill the morphologic criteria of ISC. They also exhibit crenation as a consequence of metabolic depletion, as do normal red cells which have been treated in the same way (Fig. 1B). But no confusion is possible between the long and sharp extensions of ATP-depleted, reoxygenated sickle cells and the short and round protrusions of depleted normal erythrocytes. The formation of ISC in this system could be inhibited if additives were used which enable the cells to maintain their ATP levels (Table 2 and Fig. 1C). For example, 30 m*M* adenosine prevented the decrease of red cell ATP concentration during the incubation of normal and hemoglobin-S cells. The two experiments shown in Table 2 demonstrate that sickle erythro-

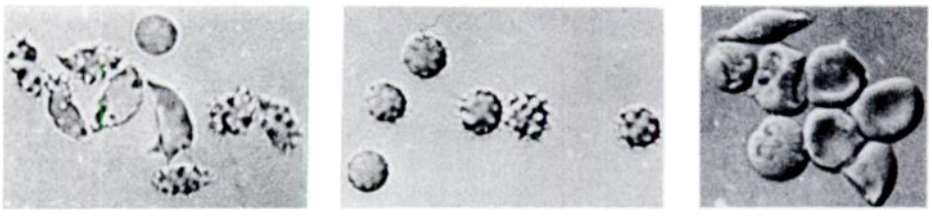

Fig. 1. Morphology of red cells after prolonged incubation under nitrogen and subsequent reoxygenation (for details, see Materials and Methods section). Nomarski optics. (A) Hb S-erythrocytes without additive. (B) Hb A-erythrocytes without additive. (C) Hb S-erythrocytes with adenosine (30 mM).

Table 2. Inhibition of ISC Formation and Maintenance of ATP by Adenosine (30 mM)

Experiment Number		Gas	Additive	% ISC	% ISC Produced	ATP mM/liter Cells
	Before incubation			21.5		1.18
	After incubation	O_2		22.6	1.4	0.16
1	After incubation	N_2		64.7	55	0.07
	After incubation	N_2	Adenosine	16.8	0	1.37
	Before incubation			25.9		1.17
	After incubation	O_2		26.6	1.0	0.21
2	After incubation	N_2		63.7	50	0.0
	After incubation	N_2	Adenosine	21.7	0	1.20

Cells incubated for 24 hr under N_2 + 5% CO_2.

cytes incubated in the presence of adenosine, while maintaining their ATP, could be unsickled by reoxygenation, whereas a considerable proportion of ATP-depleted sickle cells had been converted to ISC. Incubation under 0_2 without nutrient did not produce ISC even though ATP depletion did occur.

That the formation of ISC is related to the decline of ATP was shown by the following experiments: Sickle red cells were incubated under nitrogen in the presence of glucose, adenosine, or sodium fluoride, or in the absence of any additives. The rate of ISC formation was then related to the rate of change of red cell ATP. Figures 2A and 2B demonstrate a correlation between the decrease in ATP and the formation of ISC. Both glucose and adenosine, which delay the fall of ATP, also delayed formation of ISC. In contrast, in the sodium fluoride incubation, ATP dropped promptly, and ISC began to appear earlier than was observed when the cells were incubated with no additive.

Since there is evidence that ATP depletion enhances membrane stiffness by permitting Ca^{++} to interact with certain membrane constituents,[8] the following experiments were performed to examine the potential role of calcium in the genesis of ISC: Twenty-four-hour incubations of sickle cells were performed under nitrogen in a calcium-free medium containing 0.1 m*M* EDTA. Table 3 shows that there were almost no ISC generated under these circumstances.

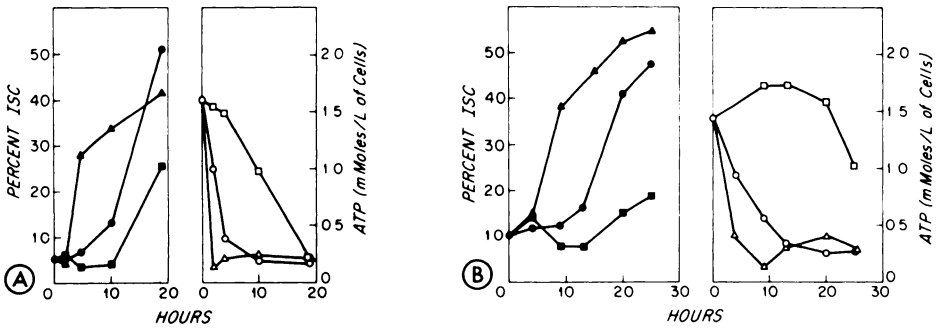

Fig. 2. (A) Percentage of ISC and ATP concentrations in sickle cell anemia blood incubated without additive (●—●, ○—○), with 15 mM glucose (■—■, □—□), and with 10 mM Na F (▲—▲, △—△). Representative of two experiments. (B) Percentage of ISC and ATP concentrations in sickle cell anemia blood incubated without additive (●—●, ○—), with 30 mM adenosine (■—■, □—□), and with 10 mM Na F (▲—▲, △—△).

Table 3. Inhibition of ISC Formation by the Absence of Ca^{++} in the Incubation Buffer

Incubation Medium	% ISC Produced Experiment Number 1	2	3	ATP (mM/liter Cells) Experiment Number 1	2	3
KHB	31.5	16.3	28.0	0.29	0.0	0.05
Ca-free KHB 10^{-4} M EDTA	6.9	0.0	0.6	0.0	0.14	0.0
KHB, 30 mM adenosine	3.6	0.0	—	1.22	0.80	—
Ca-free KHB 30 mM adenosine	2.2	—	—	1.79	—	—

Cells incubated for 24 hr under N_2 and 5% CO_2.

despite the fact that ATP was very low. A significant increase occurred in calcium containing KHB. The data in Table 3 indicate that metabolic depletion of sickle cells in KHB even in the presence of calcium produced less ISC compared to the relative proportions of ISC produced after incubation of sickle cells in dilute serum (Table 1). Addition of EDTA to serum (1 m*M* final concentration) did not reduce ISC formation, and even 5 m*M* EDTA was only slightly effective when serum was present in the incubate (Table 4).

Experiments were performed to investigate the possibility that fragmentation, which has been observed to occur during unsickling[5,6] might contribute to the formation of ISC. Hemoglobin-S erythrocytes were suspended in glucose-supplemented plasma and alternately equilibrated with O_2 + 5% CO_2 and N_2 + 5% CO_2 in round-bottom tonometer flasks at 37° C. Ten complete sickling-unsickling cycles were then induced. In five experiments of this kind, the time of exposure to each gas per cycle varied between 12 and 45 min from one experiment to another, but was constant within one experiment. Oxygen tensions were over 300 torr after each exposure to oxygen and below 5 torr after each exposure to nitrogen. No free hemoglobin was found in the plasma during the course of these experiments. Measurements of the percentage of sickled forms after each exposure to nitrogen revealed that more than 80% of the cells were sickled. The percentage of sickled forms after each oxygen period never increased and was always equal to the number of ISC present before the experiment. Table 5 shows a representative experiment which indicates that sickling-unsickling of fresh, ATP-rich red cells, alone does not produce ISC. The percentage of ISC was counted after every second reoxygenation, and remained unchanged. Table 5 also demonstrates that the cells did not lose phospholipid during the sickling-unsickling cycles. This indicates that if fragmentation occurred, it was symmetrical, involving membrane and cytoplasm as well. In addition, no correlation could be found between the artificial generation of ISC

Table 4. Effect of EDTA on ISC Formation in Dilute Serum

Incubation Medium	% ISC Produced	ATP (mM/liter Cells)
KHB: Serum 1 : 1	54	0.05
Same, + 10^{-3}M EDTA	55	0.0
Same, + 5 × 10^{-3}M EDTA	36	0.26

Cells were incubated for 24 hr under N_2 and 5% CO_2.

Table 5. Effect of Repeated Sickling and Unsickling on the Percentage of ISC and the Lipid Phosphorus Content

Deoxygenation Reoxygenation Cycles	% ISC	Lipid-Phosphorus (γ/ml Cells)
0	20.2	201.2
4	20.8	206.4
6	19.0	212.4
8	22.0	212.4
10	22.0	216.7

The cells were alternately equilibrated with nitrogen and oxygen. Time of exposure to each gas per cycle: 15 min. For further details, see text.

and the loss of membrane phospholipids. Table 6 shows a similar decrease in the amount of lipid phosphorus after a 24-hr incubation, whether ISC were generated or not. Normal hemoglobin-A red cells, hemoglobin-S red cells under oxygen, and hemoglobin-S red cells under nitrogen plus 30 m*M* adenosine, all lost small amounts of phospholipids in the absence of ISC production.

In the lower part of Table 6, experiments are shown in which ISC were formed; the loss of phospholipids from the cells was not greater under these circumstances.

DISCUSSION

ISC exhibit a variety of properties in addition to their shape, which distinguish them from other Hb S erythrocytes, and which have been held responsible for their generation. Among these are a relatively low proportion of Hb F[12] and a high density (high MCHC). Although these properties conceivably render Hb S erythrocytes more susceptible to deoxygenation induced sickling, they do not explain why in ISC, the elongated sickle shape persists upon reoxygenation.

Artificial production of ISC, as first reported by Shen,[7] was therefore used to approach the problem of ISC formation. Additives were used during prolonged incubations that enhanced or delayed the fall of the intracellular ATP concentration. These experiments demonstrated an inverse correlation between intra-

Table 6. Changes in Red Cell Phospholipid During Prolonged Incubation

Hb Type	Gas	Additive	% ISC Produced	% Phospholipids Lost
A	Air		0	12.1
A	Air		0	10.4
A	Air	Adenosine	0	7.0
S	O_2		0	3.4
S	N_2	Adenosine	0	3.0
S	N_2		38	12.8
S	N_2		38	8.7
S	N_2		41	4.1
S	N_2		52	3.3
S	N_2		53	10.0
S	N_2		54.5	9.3
S	N_2		64	6.9

Red cells were incubated for 23 ± 1 hr. Upper part: various conditions without ISC production. Lower part: Conditions under which ISC were produced.

cellular ATP and ISC formation in deoxygenated hemoglobin-S erythrocytes in vitro. It does not necessarily follow that ATP itself is the responsible compound, but at least its concentration is a sensitive indicator of a metabolic state of the cell that favors or inhibits ISC generation. A similar relationship has been found by Weed[8] and his coworkers between ATP and the deformability of hemoglobin-A red cells. These workers demonstrated increasing membrane stiffness when ATP was depleted. The membrane stiffening may play a role in the artificial generation of ISC. Exhaustion of ATP stores in the sickled state may result in a rigid cell that is unable to resume a normal shape, in spite of subsequent depolymerization of hemoglobin-S by oxygenation.

The mechanism of action of ATP or the energy state of the cell in prevention of membrane stiffening or ISC formation is not clear. Weed and his co-workers proposed that ATP may act as a calcium chelator. They found a correlation between the calcium content of red cell ghosts and rigidity, and concluded that ATP prevents calcium accumulation and thereby stiffening of the membrane. Our experiments suggest a similar relationship. Metabolic depletion of sickle cells in KHB and in the sickled state failed to produce ISC when 10^{-4} molar EDTA and no calcium were added to the incubation buffer. It should be noted, however (Table 3), that in KHB which did contain calcium, consistently smaller amounts of ISC were produced than in dilute serum. Other, as yet undetermined factors in serum may contribute to the membrane alteration that results in ISC formation. Among these factors might be lysolecithin which induces formation of highly undeformable ecchinocytes, the development of which is dependent on intracellular ATP levels.[13]

Our results, as well as those of others, indicate that fragmentation during unsickling or loss of membrane phospholipids are not responsible for ISC formation. Deoxygenated sickled erythrocytes may be subject to fragmentation in vivo by at least two mechanisms: First, detachment of little hemoglobin-containing spherules from sickled red cells can occur during unsickling. Second, the long, thin extensions of such cells are easily broken mechanically and may therefore break off during the passage through the microvasculature.[6] It should be emphasized, however, that when sickling is induced by deoxygenation and spicules are cut off by means of a laser beam,[14] the remaining cell adopts a spheroidal and not a sickled form. Our results are consistent with these observations. Multiple sickle-unsickle cycles did not increase the percentage of ISC. We have no proof that fragmentation did occur during this procedure, but if it did, it must have been of the symmetric variety, involving membrane and cytoplasm to the same extent. The fact that the amount of phospholipids per milliliter of cells remained unchanged indicates that no disproportionate membrane loss took place. This result is supported by the observation of Padilla et al.[5] that the fragments which were separated from sickled erythrocytes during reoxygenation contained hemoglobin.

Small disproportionate losses of membrane phospholipids were caused by the prolonged incubation system, but no difference was found whether ISC were produced during the incubation or not. No correlation could be detected between phospholipid decrease and the amount of ISC generated. We conclude that at least in vitro unsickling-induced fragmentation is either of minor importance or is symmetrical, and does not contribute to the generation of ISC.

Presently it cannot be decided whether ATP-dependent membrane stiffening is the main factor responsible for ISC generation in vivo. ATP depletion probably occurs in significant parts of the red cell population in SCA, when these cells sickle in areas of the microvasculature with low oxygen tension. They may thereby become trapped in an environment where plasma flow and supply of metabolic substrates almost cease, resulting in a decrease of the ATP concentration and rigidity of the membrane.

It seems possible therefore that the ISC specific lesion is linked to metabolic depletion, since our experiments have demonstrated that such a relationship does exist in vitro.

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