RESTORATION OF THE DEFORMABILITY OF "IRREVERSIBLY" SICKLED CELLS BY PROCAINE HYDROCHLORIDE

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SUMMARY: Irreversibly sickled red cells may be produced *in vitro* by incubating deoxygenated sickle cells at 37°C. The deformability of oxygenated irreversibly sickled cells produced in this way may be restored by pre or post treatment with 0.09M procaine hydrochloride. The loss of deformability associated with metabolic depletion of normal erythrocytes *in vitro* is minimized in the presence of 0.006M procaine hydrochloride. Displacement of membrane-bound calcium by procaine is suggested as a possible mechanism for the observed effects.

INTRODUCTION

Although the occurrence of irreversibly sickled cells (ISC) in people with sickle cell anemia was recognized at an early date[1,2], only recently has it been demonstrated by Serjeant et al.[3] that there is a direct correlation between the numbers of irreversibly sickled cells and a shortening of the chromium 51 red cell survival time in patients. Furthermore, abnormalities in the red cell membrane play a key role in determining whether a deoxygenated sickled cell will revert to a biconcave disk on reoxygenation[4,5]. Inasmuch as electron microscopic studies have indicated[6] that oxygenated ISC show little evidence of hemoglobin polymerization, cell rigidity cannot be attributed to altered hemoglobin. (Membrane properties of ISC cells may, however, be dependent on an interaction between membrane and hemoglobin.) Fragmentation as a result of repeated sickling-unsickling episodes has been suggested[4] as the determining factor in the origin of irreversibly sickled cells. Intraerythrocytic metabolism including an ATP-

membrane effect apparently is a factor that is independent of hemoglobin polymerization in the formation of the ISC[7]. The presumed clinical importance of ISC is considered to reside in their inflexibility which leads to difficulty in traversing the microcirculation and shortened survival time.

Recently, elevated levels of calcium have been reported[8] in SS cells and especially in ISC cells. Oxygenated SS cells contained eight times the calcium of normal AA cells, and also oxygenated ISC cells were two times richer in calcium than were oxygenated SS cells. Furthermore, because deoxygenation of SS cells augmented the permeability of the membrane to calcium, there was increased diffusion of calcium into the cell from the surrounding medium. Such calcium accumulation has been implicated in a loss of deformability when normal red cells are incubated at 37°C for 24 hours or longer in the absence of an energy source[9]. This excess calcium, which is associated with ATP depletion and a resultant non-functional calcium pump, is bound to the interior surface of the membrane and results in increased membrane stiffness. Accordingly, Eaton et al.[8], suggest that ISC formation may be due to excess calcium binding to the red cell membrane *in vivo*.

When procaine and similar cationic anesthetics bind to membranes, membrane-bound calcium[10] is displaced. Other related effects[11] of the cationic anesthetics on erythrocytes are an increased membrane area, a protection against osmotic hemolysis, an increase in membrane fluidity, and shape changes. In normal red cells, cationic agents induce a reversible change in shape from biconcave to cup-like, whereas anionic agents induce a crenated shape[12].

It is the purpose of this preliminary report to show that the membrane rigidity which is associated with ISC *in vitro* is reduced in the presence of a cationic anesthetic, procaine hydrochloride,

and that ISC, once formed *in vitro*, may be made deformable by the same drug.

MATERIALS AND METHODS

Blood was obtained from 10 patients with sickle cell anemia, nine of whom were clinically asymptomatic at the time of blood withdrawal. Diagnosis of sickle cell anemia was based on the association of clinical manifestations with the hematological parameters of a poorly compensated hemolytic anemia, demonstration of the sickling phenomenon, hemoglobin electrophoresis on cellulose acetate, and the presence of an insoluble hemoglobin in phosphate buffered dithionite solutions. Hemoglobin F, as determined by the Singer alkali denaturation method, was less than 10% in all patients. The method we used for formation of irreversibly sickled cells *in vitro* was similar to that described by Shen et al.[2], namely sterile incubation of the deoxygenated cells at 37°C. Procaine hydrochloride (Matheson, Coleman and Bell or Rom-Amer Pharmaceuticals, Ltd.*) was dissolved in tris buffer (0.147M NaCl; 0.002M $CaCl_2$; 0.012M tris (hydroxymethylaminomethane); pH 7.4 to 25% w/v. A 0.02 ml volume of this solution was added to 0.2 ml of whole blood, so that the cell suspension was 0.09M in procaine hydrochloride. This cell suspension as well as a control suspension without procaine hydrochloride was kept at 5°C for 2-12 hours before deoxygenation, which was achieved by adding two volumes of 2% sodium metabisulfite in distilled water to one volume of cell suspension. The deoxygenated cell suspensions were then incubated in sealed test tubes at 37°C for 4 hours. At the end of the period of incubation nearly 100% of the cells in both control and procaine-containing suspensions had sickled. In order to reoxygenate the suspensions, the cells were centrifuged, resuspended in 10 ml of

*Gerovital H3

TABLE 1

Restoration of deformability of ISC by procaine hydrochloride

SAMPLE	VOLUME COLLECTED, ML.			
	0.2	0.4	0.5	1.0
SS + O_2	3	7	9	25
SS + O_2 + procaine	3	6	8.5	22
ISC + O_2	12	23	32	101
ISC + O_2 + procaine	6	12	18	49

Filtration time in sec. of oxygenated SS cells, with and without 0.09M procaine; and of oxygenated irreversibly sickled cells with and without 0.09M procaine. Nuclepore pore size 5 μm; 3 ml starting volume with hydrostatic driving pressure.

fresh tris buffer and exposed to air. This procedure was repeated 3 times so as to effectively eliminate bisulfite. The final concentration of cells was adjusted to 0.25% v/v for deformability measurements and phase microscopy.

As a test for cell deformability the cell suspensions were filtered through Nuclepore polycarbonate filters with 5 μm pore size (Nuclepore Corp., Pleasanton, Calif.). Three ml of cell suspension was allowed to pass vertically through the filter and flow rates were measured by noting collection times versus volume collected. The hematocrit of the filtered suspension was equal to the starting hematocrit, i.e. cell concentration was the same on both sides of the filter. The filters were ultrasonicated in tris buffer before use so as to remove trapped air. A new filter was used for each determination.

TABLE 2

Reversal of sickle cell shape with time after start of oxygenation

t hr.	Percentage of sickled cells 0.09M procaine	control
0	88	95
1	45	95
2	40	96
3	33	95
20	30	92

Time course of reversal of *in vitro*-formed ISC by procaine hydrochloride in a typical experiment. Percentage of remaining deformed cells is tabulated as a function of time in hours after removal of reducing agent and exposure to room air. $p < 0.001$

RESULTS

Table 1 summarizes the filtration data for oxygenated ISC with and without procaine. As an additional control, the filter times of freshly drawn oxygenated SS blood with and without procaine were also measured and found to be nearly identical. This finding eliminates the possibility that procaine affects the filter time by merely changing the cell volume or shape.

Additional evidence for restoration of membrane flexibility is seen in Table 2, which summarizes changes in shape with time after the elimination of the reducing agent. Ninety five percent of the control cells were still sickled after 3 hours, whereas only 33% of cells which had been treated with procaine were still sickled, i.e. approximately 62% of the treated cells had changed shape. Table 3 summarizes the data on shape reversal for 5 patients. In each of the above experiments procaine was added before

TABLE 3

Reversal of sickling in samples from five patients

Patient	Percent Reversal	
	Procaine Sample	Control
1	71	9
2	61	5
3	72	2
4	70	1
5	63	2

Percentages of cells which reversed shape in procaine-treated sample and control in blood of 5 patients after 3 hours oxygenation. Percentages based on counts of 300 or more cells for each determination. $p < 0.001$

deoxygenation. The reversibility of ISC preformed *in vitro* was tested by a similar protocol, except that 0.09M procaine hydrochloride was added *after* the deoxygenation period - at the time of oxygenation. One hour after oxygenation had commenced, 90% of the control cells were still sickled, whereas 80% of the procaine-treated cells had reversed in shape.

The hypothesis that procaine hydrochloride competes with calcium for membrane binding sites and may displace calcium from the membrane is made plausible by filtration data on normal AA red cells which had been metabolically depleted at 37°C for 20-48 hours. Freshly drawn normal AA cells were washed in tris buffer and diluted to 0.25% v/v, with and without added procaine. After 20-48 hours incubation at 37°C, the cell suspensions were filtered through Nuclepore filters with 3 μm pore size. Table 4 shows that in the absence of procaine (control sample) the cell rigidity increased in 20 hours by a factor of 23:1 when compared with normal fresh AA

TABLE 4

Effect of procaine hydrochloride on normal erythrocyte deformability

Blood Sample	Incub. time, hr.	Volume Collected, Ml 0.1	0.2	0.3	0.5	1.0	t/t_0 for 1 ml
Control	0	3	5	9	16	39	1.0
Control	20	15	36	79	300	900	23
Control	48	1020	-	-	-	-	-
Procaine	20	3	7	12	21	54	1.4
Procaine	48	8	9	15	32	103	2.6

Normal red cell deformability before and after metabolic depletion with and without .006M procaine. Filtration time in seconds as a function of incubation time and volume collected. Filter flow rate reproducibility 10.8%, based on 133 determinations, 3 μm pore size. t/t_0=sample flow time/fresh blood flow time for 1 ml.

cells. The 48 hour control cells were too rigid to pass through the filter. The optimum concentration of procaine hydrochloride was found to be 0.006 M. The filter times for normal AA cells that were incubated in tris buffer containing this concentration of procaine were much shorter; at 24 hours the time was only 1.4 times as long as the time for fresh cells, and at 48 hours was only 2.6 times as long. This indicated that procaine had effectively protected the cells against the calcium accumulation and resultant rigidity which is known to occur in this situation[9], and that the membranes had thereby remained plastic. We propose that a similar mechanism may be operative in the observed restoration of deformability by procaine hydrochloride in irreversibly sickled cells.

DISCUSSION

Procaine hydrochloride, one of a large group of cationic anesthetics, has been demonstrated to have potential as a tool for controlling red cell deformability. The mechanism of action of procaine in mediating deformability is unknown; it may involve membrane-bound calcium. Calcium measurements are now in progress which should be helpful in deciding this point.

It is known[13] that procaine is rapidly hydrolyzed in plasma by cholinesterase, with two breakdown products - p-aminobenzoic acid and diethylaminoethanol. Preliminary experiments have demonstrated that p-aminobenzoic acid may be equally as effective as procaine in controlling membrane flexibility. If this is true, kinetic studies of the interactions between procaine, calcium and the cell membrane, using cholinesterase inhibitors, are clearly needed for an understanding of the mechanism of action of procaine in the presence of red cells and plasma, and to serve as a guide for the treatment of red cells *in vivo*.

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