Increased Erythrocyte Ca^{2+} Content in Hereditary Spherocytosis

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Extract

The Ca^{2+} content of red blood cells (RBC) appears to correlate inversely with the Ca^{2+} -ATPase activity of the red cell membrane (Fig. 1). When hereditary spherocytosis (HS) cells and normal control erythrocytes are compared, the dependence of Ca^{2+} content upon ATPase activity is similar. The Ca^{2+} content of HS red cells is increased compared with appropriate control erythrocytes matched for cell age (Fig. 2). Splenectomy has a different effect upon HS cells than upon normal cells (Fig. 2). Erythrocytes of splenectomized patients with HS seem to contain less Ca^{2+} than do those of presplenectomy patients with HS, whereas the reverse appears to pertain in normal subjects.

Speculation

The control of RBC Ca^{2+} content is complex. Although there may be some dependence upon Ca^{2+} -ATPase, other factors are involved. The splenic environment may be particularly unfavorable for the extrusion of Ca^{2+} from the HS red cell, thus leading to excessive accumulation of Ca^{2+} and further impairment of RBC membrane function in the splenic milieu. This process may play a fundamental role in the pathogenesis of hemolysis in hereditary spherocytosis.

Hereditary spherocytosis is thought to be the result of an intrinsic abnormality of the red cell membrane protein. The impaired deformability (16) and reduced capacity to form endocytic vacuoles (3) observed in that syndrome might be explained by an altered interaction of the RBC membrane with Ca^{2+} .

A relative deficiency of membrane Ca^{2+} -dependent adenosine triphosphatase (Ca^{2+} -ATPase) in HS ghosts has been described previously (7). This observation might be related to the pathogenesis of the HS defect in several ways. There is evidence which suggests that membrane Ca^{2+} -ATPase may be linked with the active transport of Ca^{2+} from the red cell (17, 27). Furthermore, elevation of red cell Ca^{2+} content is associated with impaired membrane deformability (31), and impaired endocytic vacuole formation (3) in normal RBC.

These studies were undertaken in an attempt to determine whether an increase in erythrocyte Ca^{2+} content was present in HS cells and how this might relate to the pathogenesis of HS.

PATIENT SELECTION

Patients were selected from the clinical services of the Departments of Medicine and Pediatrics of the UCLA Center for the Health Sciences. The diagnosis of HS was made on the basis of characteristic morphology, family history, and typical patterns of autohemolysis and osmotic fragility. Normal control subjects were selected from the clinical, laboratory, and secretarial staffs of the hospital. High reticulocyte control specimens were obtained from patients with acquired autoimmune hemolytic anemia (two patients), hypersplenism (one), and patients recovering from pernicious anemia (one), and from iron deficiency anemia (one).

Splenectomized control subjects were all patients with Hodgkin disease who had previously undergone staging laparotomy. None of these patients had ever received chemotherapy.

The ages of subjects studied was quite variable. No correlation between RBC Ca^{2+} content and the age of the subject was observed.

METHODS

Specimens were collected in preservative-free heparin (1 mg/ml). The plasma and buffy coat were carefully removed after centrifugation and the red cells were washed three times in physiologic saline buffered with Tris (pH = 7.4). A suspension of cells was prepared to have a hematocrit of 75-80%. Contamination by leukocytes was less than 500 cells/mm³.

Eight milliliters of 10% trichloroacetic acid (TCA) were added to 4 ml red cell suspension. The filtrate was saved and the proteinaceous pellet was washed twice with 4 ml TCA. The filtrate and wash solutions were combined and the TCA was removed by extracting twice with diethyl ether.

The filtrate was then lyophilized and the powder dissolved in 1 ml 0.5% lanthanum chloride in 2.5% HCl. The Ca^{2+} content of each specimen was measured with a Varian Techtron atomic absorption spectrometer (model 1200) and calculated from a standard curve, determined with each specimen.

To ensure that elevations in Ca^{2+} content were not due to trapped plasma, ghosts were prepared and the Ca^{2+} content of ghosts was compared with the Ca^{2+} content of erythrocytes. The Ca^{2+} content of the wash solutions was negligible, which indicated minimal contamination of washed red cells with trapped plasma. The Ca^{2+} content of ghosts was identical with that of whole RBC, confirming the observations of Harrison and Long (12). Known concentrations of Ca^{2+} were subjected to the extraction procedure and recovery was found to be complete.

Ca^{2+} content was expressed as a function of the volume of packed red cells. Although this allowed comparison of our data with observations of prior investigators, when the studies were analyzed, it became apparent that since Ca^{2+} is completely membrane bound, the volume of packed cells might not allow a strict comparison of spherocytes and normal erythrocytes. Although data are not available which would allow the expression of RBC Ca^{2+} content per unit RBC membrane, volume of packed cells would seem to be a more appropriate denominator than either number of cells or hemoglobin content. This method would include a factor for the reduced membrane content of a spherocyte and the increased number of cells packed in a given volume.

Ghosts were prepared (7) quantitatively from the red cells of three splenectomized patients with spherocytosis and three normal adults. Protein content was measured by the method of Lowry *et al.* (19). The membrane content of all spherocytic ghosts was lower than that of all normal ghosts (Table 1).

The glutamic oxaloacetic transaminase activity (EGOT)

Each specimen was assayed. Cells were prepared as described above and an hemolysate was prepared by a 20-fold dilution in distilled water. The stroma was removed after centrifugation and the lysate analyzed for enzyme activity by the method of Karmali *et al.* (15). This enzyme is a commonly accepted measure of mean cell age (2, 22).

Filtrates of whole fresh blood specimens were prepared in 6% perchloric acid (8) and analyzed for ATP (1). Ca^{2+} -ATPase activity of red cell membranes was measured as previously described (7).

RESULTS

The mean cell age of the high reticulocyte control group was much younger than that of the normal and splenectomized control groups as measured by EGOT activity ($P < 0.01$) (Table 2). Similarly, the presplenectomy HS group was younger than the postsplenectomy HS group ($P < 0.01$). The high reticulocyte controls appeared to be somewhat more youthful than the presplenectomy HS group (by both reticulocyte count and EGOT activity) and the EGOT activity of postsplenectomy HS cells was higher than that of the normal controls ($P < 0.01$). The ATP content of the high reticulocyte control and the presplenectomy HS groups appeared to be slightly higher than that observed in the other groups, but these differences were not significant.

The relationship of red cell Ca^{2+} content to membrane Ca^{2+} -ATPase was examined by measuring these parameters on the same blood specimen in 9 patients and 22 control subjects (Fig. 1). The data were analyzed using computer program BMDP1V (one-way analysis of variance and covariance, version of February 7, 1975 (5)). The slopes of the regression lines for HS and control subjects were not significantly different ($P > 0.10$), but the y intercepts were different ($P < 0.001$). The slope of the combined regression line was significantly different from 0 ($P < 0.05$).

The Ca^{2+} content of red cells from splenectomized HS patients was higher than that of normal control erythrocytes ($P < 0.001$) (Fig. 2). The Ca^{2+} content of normal erythrocytes was similar to

previously reported values (6, 12, 18). The level of Ca^{2+} in cells of presplenectomy HS patients was higher than that in the high reticulocyte control cells ($P < 0.005$). Curiously, splenectomy appeared to have opposite effects on the Ca^{2+} content of spherocytic red cells as compared with normal controls.

The Ca^{2+} content of HS erythrocytes was slightly lower in splenectomized patients, whereas the Ca^{2+} content of hematologically normal red cells was slightly higher in splenectomized subjects. The effect of splenectomy on the Ca^{2+} content of normal control and HS red cells was contrasted by the formula

$$t = \frac{(\bar{X}_1 - \bar{X}_2) - (\bar{X}_3 - \bar{X}_4)}{\text{Sp} \sqrt{\frac{1}{n_1} + \frac{1}{n_2} + \frac{1}{n_3} + \frac{1}{n_4}}}$$

for $n_1 + n_2 + n_3 + n_4 - 4$ degrees of freedom, where

$$\text{Sp} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + (n_3 - 1)s_3^2 + (n_4 - 1)s_4^2}{n_1 + n_2 + n_3 + n_4 - 4}}$$

The effect of splenectomy was thus found to be significantly different ($P < 0.05$).

Four HS subjects were studied before splenectomy and again at least 3 months after splenectomy (Table 3). In all patients the Ca^{2+} content of HS erythrocytes was lower after splenectomy, although the difference was insignificant in patient 4. In each patient, the EGOT activity was lower after splenectomy. This confirms greater mean cell age, *i.e.*, longer RBC survival, in HS patients after splenectomy. Unfortunately, the opportunity to study control patients before and after splenectomy did not present itself.

DISCUSSION

The present studies demonstrate increased Ca^{2+} content of HS red cells. The clinical severity of hemolysis was quite similar in all HS patients examined while the observed Ca^{2+} content in each study group was variable. The variability of erythrocyte Ca^{2+} content has been noted by several other groups (6, 12, 18). Even though a few patients with HS had red cell Ca^{2+} values within the normal range, no correlation was possible between the clinical severity of hemolytic anemia in a given patient, and the Ca^{2+} content of that patient's RBC.

Harrison and Long (12) have shown that red cell Ca^{2+} is completely bound to the membrane. Unfortunately, these studies do not express RBC Ca^{2+} content as a function of membrane content. Since the membrane protein content of a given volume of spherocytes is equal to, or less than that of the same volume of normal erythrocytes (Table 1), the increased Ca^{2+} content observed in spherocytes might, in fact, be even more striking.

Accumulation of Ca^{2+} by normal erythrocytes as a result of metabolic depletion is associated with reduced membrane deformability (31) and an inhibition of membrane endocytic vacuole formation (3). These two functions are impaired in HS membranes (16, 28). Normal erythrocytes also demonstrate a dramatic loss of K^+ when Ca^{2+} content increases (31). This is not a feature

Table 1. Membrane protein content of spherocytic and normal red blood cells (RBC)

	mg protein content
	mg RBC
Normal RBC	
Subject 1	4.9
Subject 2	6.2
Subject 3	5.3
Spherocytic RBC	
Subject 1	2.9
Subject 2	3.6
Subject 3	3.5

Table 2. Characteristics of red blood cell (RBC) samples

No.	Reticulocyte count, %	EGOT activity, IU/10 ¹⁰ RBC/min	ATP, mmol/liter cells
Control subjects			
Normal	15	0.49 ± 0.04	1.11 ± 0.08
High reticulocyte	5	18.2 ± 3.1	1.54 ± 0.16
Splenectomized	5	0.47 ± 0.03	1.39 ± 0.17
HS patients			
Presplenectomy	11	13.0 ± 0.69	0.98 ± 0.07
Splenectomized	11	0.68 ± 0.06	1.20 ± 0.07

¹ Values are expressed as means ± SE. EGOT: glutamic oxaloacetic transaminase; HS: hereditary spherocytosis.

Fig. 1. The relationship of red blood cell (RBC) Ca^{2+} ATPase and Ca^{2+} content. Each point represents analysis of a single blood specimen for both parameters. $\times$: normal subject ($n = 14$); $\bullet$: high reticulocyte control subjects ($n = 3$, including two subjects with autoimmune hemolytic anemia); $\blacktriangle$: splenectomized control subjects ($n = 5$); $\circ$: presplenectomy hereditary spherocytosis (HS) ($n = 9$); $\triangle$: postsplenectomy HS ($n = 6$). Because of the small number of high reticulocyte and splenectomized control subjects, all controls were analyzed together. Similarly, all HS subjects were grouped for analysis. Computer analysis (see text) assigned the following regression lines: for HS subjects, $y = 2.05 - 0.0008x$ (—); for normal subjects, $y = 0.986 - 0.0002x$ (---).

Fig. 2. Red blood cell (RBC) Ca^{2+} content in hereditary spherocytosis. Data are expressed as the mean $\pm$ SE. NL: normal RBC; H.R.: high reticulocyte control RBC; SPLX: postsplenectomy control RBC; H.S.P.S.: presplenectomy hereditary spherocytes; H.S. SPLX: postsplenectomy hereditary spherocytes; N: number of specimens examined.

of HS cells. In fact, HS cells show a marked permeability to Na^+ (4). The reason for this is not known. However, in contrast to metabolically depleted cells, HS erythrocytes demonstrate increased Ca^{2+} content in association with normal or increased ATP.

Ca^{2+} accumulation has also been observed in sickle erythrocytes, especially irreversibly sickled cells (ISC) (6). This phenomenon may relate to the marked loss of K^+ and water by the ISC (29, 30), and to the loss of membrane deformability. It may not be critical, however, to the formation of the ISC, since irreversible sickling may occur in the presence of adequate cellular ATP and a

Table 3. Calcium content of hereditary spherocytosis red blood cells (RBC) before and after splenectomy¹

Subject	Presplenectomy		Postsplenectomy	
	Ca^{2+} content, $\mu\text{g/ml RBC}$	EGOT activity, IU/ 10^{10} RBC/min	Ca^{2+} content, $\mu\text{g/ml RBC}$	EGOT activity, IU/ 10^{10} RBC/min
Patient 1	1.46	1.03	0.69	0.26
Patient 2	2.30	1.17	1.88	0.66
Patient 3	1.65	0.98	0.65	0.57
Patient 4	1.29	1.32	1.27	0.41

¹ EGOT: glutamic oxaloacetic transaminase.

low Ca^{2+} content (9). Furthermore, maintenance of the ATP content of sickle cells protects against Ca^{2+} accumulation with deoxygenation, but does not prevent formation of the ISC (25). Thus, in the sickle model, Ca^{2+} accumulation seems to relate to metabolic inadequacy, whereas in the HS cell, Ca^{2+} accumulates in the presence of apparently adequate ATP stores.

Although the present data are consistent with an inverse correlation between membrane Ca^{2+} -ATPase and red cell Ca^{2+} content (Fig. 1), the control of red cell Ca^{2+} is apparently more complex. The data suggests that for any given value of Ca^{2+} -ATPase, the Ca^{2+} content of an HS red cell would be greater than that of a normal erythrocyte.

There are two possible mechanisms by which an elevated RBC Ca^{2+} content might be related to the pathogenesis of the HS defect. Since "muscle-like" proteins have been described in the red cell membrane (21, 23, 26), one might speculate upon the role of such components in the preservation of membrane deformability. The membrane has a contractile capacity which probably derives its energy from a Ca^{2+} -ATPase (24, 32). Jacob and his coworkers (13, 14) have demonstrated abnormalities in extracts of HS membranes which contain the presumed contractile elements. The re-

relationship between the membrane contractile proteins, Ca^{2+} content, and the pathogenesis of the HS defect is unclear. Greenquist and Shohet (11) have described impaired phosphorylation of spectrin in the red cells of subjects with HS. In normal membranes, they found that this protein kinase activity was inhibited by elevated Ca^{2+} concentration (10). The data of Lux and John (20) suggest that phosphorylated spectrin might correlate with a relative state of deformability, whereas nonphosphorylated spectrin might be associated with a state of the membrane which is less deformable. Thus, the observed elevation of red cell Ca^{2+} in HS might be a pathogenetic link between the relative deficiency of Ca^{2+} -ATPase (7) and the described impairment of membrane deformability.

SUMMARY

The data demonstrate an increased Ca^{2+} content of HS red cells compared with appropriate control erythrocytes matched for mean cell age. Investigation of the dependence of red cell Ca^{2+} content upon Ca^{2+} -ATPase activity suggests that for any given ATPase activity, an HS cell has a higher Ca^{2+} content than a normal cell. The different effect of splenectomy on the Ca^{2+} content of HS and normal cells suggests that the control of red cell Ca^{2+} is more complex.

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