Increased Red Cell Calcium, Decreased Calcium Adenosine Triphosphatase, and Altered Membrane Proteins During Fava Bean Hemolysis in Glucose-6-Phosphate Dehydrogenase–Deficient (Mediterranean Variant) Individuals

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RBCs from four glucose-6-phosphate dehydrogenase (G6PD)-deficient (Mediterranean variant) subjects were studied during fava bean hemolysis. In the density-fractionated RBC calcium level, Ca^{2+}-ATPase activity, reduced glutathione level, and ghost protein pattern were studied. In the bottom fraction, containing most heavily damaged RBCs, calcium level ranged from 143 to 244 μmol/L RBCs (healthy G6PD-deficient controls: 17 ± 5 μmol/L RBCs). The Ca^{2+}-ATPase activity ranged from 0.87 to 1.84 μmol ATP consumed/g Hb/min (healthy G6PD-deficient controls: 2.27 ± 0.4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ghosts showed: (1) the presence of high mol wt aggregates (in three cases they were reduced by dithioerythritol; in one case, only partial reduction was possible); (2) the presence of multiple, scattered new bands; and (3) the reduction of band 3. Oxidant-mediated damage to active calcium extrusion, hypothetically associated with increased calcium permeability, may explain the large increase in calcium levels. They, in turn, could activate calcium-dependent protease activity, giving rise to the profound changes in the ghost protein pattern.

DURING FAVA bean hemolytic crisis in glucose-6-phosphate dehydrogenase (G6PD)-deficient (Mediterranean variant) individuals, a number of biochemical, morphological, and rheological modifications occur in the red cells (RBCs): eg, reduced glutathione (GSH), NADPH, and NADH are oxidized[1]; methemoglobin and Heinz bodies are formed[2,3]; and severely deformed, rigid RBCs are present, with tightly bonded opposing membrane areas. Such membrane cross-linked, or cross-bonded,[4,5] RBCs may represent more than 50% of the total RBC population during the crisis and are rapidly cleared from the circulation.[4,5]

A completely similar picture was obtained when such RBCs were treated with 0.5 to 1 mmol/L divicine or isouramil, oxidant pyrimidine derivatives present in high amounts in fava beans.[6-8] The analogy between the crisis protein pattern and that observed in RBCs containing high calcium levels[9] and the increase in RBC calcium after oxidant treatment,[10] led us to assay calcium level and Ca^{2+}-ATPase activity in density-fractionated RBCs during the favic crisis in four subjects. A remarkable increase in RBC calcium level and decreased Ca^{2+}-ATPase activity were found in all cases. Moreover, the membrane protein pattern was markedly altered: band 3 decreased, and a high mol wt aggregate was constantly present.

MATERIALS AND METHODS

Materials. Enzymes and biochemicals were obtained from Boehringer Mannheim (Tutzing OBB, FRG); bovine serum albumin (BSA) (fraction V) and saponin from Sigma Chemical Company (St Louis); and Chelex-100 and electrophoresis reagents from Bio-Rad Laboratories (Richmond, Calif). All other chemicals were Merck (Darmstadt, FRG) products of reagent grade.

Blood samples. Four cases representative of a series of 20 favic crises were the object of this study. The patients were investigated during acute hemolysis, as documented by high counts of cross-linked RBCs, high amounts of Heinz bodies, massive hemoglobinuria, and low hematocrit values (Table 1). The patients were admitted 20 to 48 hours after eating undefined amounts of fava beans. Control blood was obtained from healthy G6PD-deficient or from normal adult males. Hemolytic and control subjects were of Sardinian ancestry. Informed consent was obtained from all subjects according to the principles of the Helsinki declaration.

On admission of the patients, blood was obtained by venipuncture into heparinized Vacutainers (Becton Dickinson, Rutherford, NJ), chilled on crushed ice, and immediately processed. Microhematocrit (Htc) and determination of cross-linked RBCs, Heinz bodies, and G6PD activity were performed on unfractionated RBCs. After removal of buffy coat by aspiration, RBCs were density-separated according to the procedure of Murphy,[11] modified in that separation by centrifugation was done at 1,000 g for 120 minutes at 30 °C. The top 20% (briefly: top) and bottom 20% (briefly: bottom) were harvested by slow aspiration through a capillary. The packed RBCs were washed three times with ice-cold, calcium-free NaCl. The washed RBCs were used immediately for Ca^{2+}-ATPase and pyruvate kinase (PK) activity assay, GSH determination, and ghost preparation, or were packed and frozen at −80 °C for calcium assay. Calcium assays were performed eight weeks later.

Biochemical assays. Preparation of hemolysates from leukocyte-free RBCs and measurement of G6PD and PK activity were performed according to Beutler.[12] Heinz bodies were measured turbidimetrically.[13] GSH was assayed according to Beutler,[12] Ca^{2+}-ATPase was measured according to Maretzki et al[14] with the following modifications: 5 μL packed RBCs were added to 1.5 mL of a prewarmed solution containing 120 mmol/L KCl, 20 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.01 mmol/L $CaCl_2$, 10 mmol/L imidazole, 0.65 mmol/L phosphoenolpyruvate, 2 mmol/L ATP, 0.30 mmol/L NADH, 0.02% saponin, 20 IU lactate dehydrogenase, and 10 IU pyruvate kinase. Decrease in optical density was followed at 366 nm and 37 °C. A second assay was run by the same procedure, using the same solution supplemented with 0.5 mmol/L lanthanum, which specifically inhibits Ca^{2+}-ATPase activity.[15] The share of the Ca^{2+}-ATPase was obtained by difference. To assay calcium concentration, 0.5 mL washed, packed RBCs were lysed with 1 mL water and mixed with 4 mL of 10% (wt/vol) trichloroacetic acid (TCA) containing 20,000 ppm (wt/vol) lanthanum. Calcium was determined in the TCA/lanthanum supernatants by absorption atomic

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Table 1. Heinz Bodies, Cross-linked RBC, Calcium Levels, Ca^{2+}-ATPase, and GSH Levels in Four Favic Crises

Patient (Age, Sex)	G6PD* Activity	Htc	Heinz† Bodies	Cross-linked‡ RBC	Fraction	Calcium§ Level	Ca^{2+}-ATPase* Activity	GSH§ Level	PK* Activity
D.I.	2.31	32	367	15	Top	29	4.21	1,126	279
(72, M)					Bottom	143	1.84	342	34.2
C.G.	1.05	29	500	55	Top	—	3.71	406	48
(42, M)					Bottom	244	0.99	113	24
B.P.	3.91	20	454	57	Top	58	3.61	1,308	79
(70, F)					Bottom	212	1.52	600	21
M.A.	1.52	20	1,280	78	Top	85	3.12	812	50.3
(6, M)					Bottom	149	0.87	298	20.1
G6PD-deficient,	0.35 ± 0.4	43 ± 5	100 ± 10	<1	Top	18 ± 5	2.72 ± 0.6	1,433 ± 153	45 ± 6
Healthy controls					Bottom	17 ± 5	2.27 ± 0.4	1,243 ± 110	19 ± 7
(18–45, M)	N = 7	7	7	7		7	7	7	5
Normal controls	8.73 ± 1.8	46 ± 3	110 ± 9	<1	Top	16 ± 4	2.48 ± 0.6	2,016 ± 175	50 ± 6
(28–48, M)					Bottom	18 ± 6	2.27 ± 0.6	1,750 ± 251	21 ± 7
	N = 7	7	7	7		7	7	7	5

Mean values SD; N, number of subjects.

*μmol substrate transformed/g Hb/min (37 °C).

†Percentage of the turbidity of G6PD-deficient, healthy controls.

‡Percentage of RBC population.

§μmol/L RBCs. Hemoglobinuria (g/dL, standard cyanmet-Hb technique: D.I., 0.4; C.G., 0.22; B.P., 0.8; M.A., 0.86).

spectrophotometry according to Eaton et al.[16] To minimize calcium contamination, all solutions were prepared with Millipore Milli-Q purified water and filtered through a Chelex-100 ion exchange resin. RBC ghosts were prepared by hypotonic lysis at 0 °C.[17] SDS-PAGE of membrane samples was performed on 4% acrylamide gels.[18]

Cross-linked RBCs. The percentage of cross-linked RBCs was determined by microscopic examination (Zeiss optics, interference contrast) of hypotonic RBC suspensions according to Fischer et al.[4,5]

RESULTS

The basic criteria for selecting our four cases was the presence of high amounts of Heinz bodies and cross-linked RBCs. These heavily deformed cells have been the target of oxidant insult, probably brought about by the combined action of divicine + isouramil + ascorbate.[4,6–8] The presence of cross-linked RBCs indicates that hemolysis is continuing, while their absence points to a residual population of young, undamaged RBCs.[4,5] A conservative fractionation procedure was adopted in order to increase the yield of the heavier, more damaged RBCs.[11] As shown in Table 1, all parameters were more clearly modified in the bottom fractions. On average, the calcium level was increased about tenfold in the bottom fractions, and only threefold in the top fractions. The Ca^{2+}-ATPase activity was increased in the top fractions and lowered in the bottom fractions. The same trend was noted for GSH, which was almost normal in the top fraction in two patients (D.I. and B.P.) but was remarkably lower in the bottom fraction in all four cases. The top fractions were rich in young cells, as reflected by increased PK[19,20] and Ca^{2+}-ATPase[21] activity. Comparison of the four cases showed that no correlation existed in patients between the extent of calcium levels, impairment of Ca^{2+}-ATPase, the amounts of residual GSH, and the extent of Heinz bodies and cross-linked RBCs. For example, in patient C.G., the highest calcium level was accompanied by relatively low amounts of Heinz bodies and cross-linked RBCs.

SDS-PAGE of ghosts showed extensive alterations (Fig 1). In the bottom fractions, the following findings were constant:

1. The appearance of high mol wt aggregates. In some cases (Fig 1, B–B') the aggregates could be reduced by

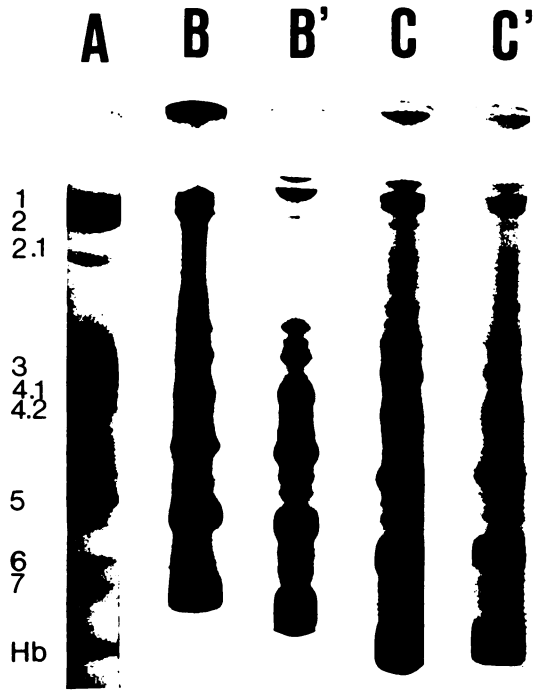

Fig 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ghosts isolated during fava bean hemolytic crisis. All ghost samples were isolated from the bottom RBC fraction. (A) G6PD-deficient control not in crisis; (B) patient B.P.; (C) patient C.G.; B', C': samples from the two patients that were reduced with 10 mmol/L DTE before electrophoresis. Dithioerythritol (DTE) reduction of the high mol wt aggregate is complete in patient B.P. (gel B'), incomplete in patient C.G. (gel C').

dithioerythritol (DTE). In other cases (Fig 1, C–C'), only a partial reduction was possible.

2. The remarkable decrease of band 3, which did not reappear after reduction with DTE.
3. The presence of multiple new bands scattered over the whole range (Fig 1, B'–C–C').

The same changes were noted after treatment of G6PD-deficient RBCs with 0.5 mmol/L divicine (not shown). These results are in agreement with recent data by Lorand et al[22] who showed the selective degradation of band 3 in calcium-enriched RBCs, as a result of calcium activation of protease activity. Calcium-mediated activation of transglutaminase may account for the formation of the nonreducible high mol wt aggregates constantly present in the G6PD-deficient RBC after 24-hour treatment with divicine (not shown) and occasionally present in the favic crisis (Fig 1, C–C'). The calcium concentrations calculated from the levels reported here for the bottom fractions range between 0.2 to 0.4 mmol/L, and are compatible with activation of latent transglutaminase activity.[23]

DISCUSSION

Passive permeability to calcium is exceedingly low in RBCs, and active extrusion is very active.[24] Therefore, a large increase in calcium influx has to be assumed in favism to explain the very high levels reported here and in the accompanying article.[25] At present, no data are available on the oxidant-mediated increase in passive calcium permeability in G6PD deficiency.

Finally, it should be remembered that β-thalassemic RBCs have large protein aggregates in the membrane[26] and increased intracellular calcium levels[27]; calcium is distinctly higher in splenectomized β-thalassemic patients, pointing to the preferential removal of high-calcium RBCs.[27] We can conclude that an oxidant-mediated increase in calcium levels in favism can activate proteolytic activities[22,28,29] and thereby induce profound changes in the membrane proteins.

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