Erythrocyte Glutathione S-Transferase Deficiency and Hemolytic Anemia

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A patient with unexplained erythrocyte glutathione-S-transferase (GST) deficiency has been detected among 513 unrelated persons with hemolytic anemia. An otherwise healthy adult male, the deficient individual had a mild hemolytic anemia with splenomegaly, indirect hyperbilirubinemia, and cholelithiasis. Because he was adopted and childless, the hereditary nature of the defect could not be established. The residual enzyme activity was only about 15% of mean normal. Depletion of glutathione (GSH) from the cells by 1-chloro-2,4-dinitrobenzene (CDNB), a substrate for GST, was somewhat decreased in the red cells from the patient, suggesting that a functional defect existed. The kinetic properties of the residual enzyme and the ratio of activity to antigenicity were normal. Modest decreases in leukocyte and platelet GST activities were documented. Although a cause-and-effect relationship between the GST deficiency and hemolysis may exist, this cannot be proven in the absence of affected family members.

More than 30 years ago decreased levels of red cell reduced glutathione (GSH) and increased sensitivity of GSH to oxidation were found to play an important role in sensitivity to drug-induced hemolytic anemia. This finding focused attention on the importance of the reduction, oxidation, and, subsequently, the synthesis of GSH in the red cell; deficiencies in all of the enzymes that catalyze these reactions have been discovered.

A different metabolic pathway that utilizes GSH, viz, the glutathione-S-transferase (GST) reaction, has been characterized in recent years. The enzyme GST conjugates GSH to electrophilic xenobiotics, forming a thioether bond and detoxifying them. First studied in liver, where GST was found to play a role in bilirubin transport as well as in conjugating substances such as sulfobromophthalain sodium (Bromsulphalein; BSP), this activity was found, about 10 years ago, also to be present in erythrocytes.

GST is, in reality, a family of enzymes controlled by different genetic loci. The enzyme in red cells, designated GST1 or GSTM1, is different from the major liver enzymes GST1 and GST2. The function of the erythrocyte enzyme is not known, but the red cell membrane contains transport system(s) that actively transport GSH-xenobiotic conjugates from the erythrocyte. Thus GST may serve to rid the red cell, and perhaps to scavenge the blood stream, of foreign molecules. Red cell GST also binds heme, an extremely hydrophobic molecule, in a manner analogous to the binding of bilirubin by liver GST isozymes. It has been suggested that its function may be the intracellular transport of heme within the developing erythroid cell.

For the past several years we have assayed the red cell glutathione-S-transferase activity of all blood samples sent to this laboratory for diagnosis of hematologic disorders with the hope that a deficient sample might shed light on the function of this enzyme in erythrocyte physiology. We have found a patient with moderately severe deficiency of the red cell enzyme, the residual activity being only about 15% of normal.

Materials and Methods

Patient Population. The red cells of 513 unrelated patients have been assayed for GST activity over the past 3 years. Over three quarters of the patients had hemolytic anemia of unknown or uncertain origin. Patients of all ages were represented, with a mean age of 21.5 years and a range from newborn to 90 years of age.

Cell Separations. Red cells were separated into a low-density, reticulocyte-rich fraction using Percoll-Renographin. Leukocytes were separated using Histopaque 1077. Platelets were obtained by isolating a platelet-rich layer by centrifugation at 400 g for 10 minutes and washing three times with 0.05 mol/L citrate solution, pH 6.2, and centrifuging at 1,500 g for 15 minutes. Fibroblasts were cultured from skin biopsies using standard methods.

Enzyme Assays. Blood samples were anticoagulated with EDTA and shipped to La Jolla on ice. Adenosine triphosphate (ATP) and 2,3-DPG estimations were carried out on perchloric acid extracts prepared from freshly drawn blood that were neutralized and sent to La Jolla on ice. Assays for metabolic intermediates and for enzymes, including GST, were performed by previously described methods.

The assay for GST is based upon the procedure described by Habig et al, which takes advantage of the fact that the conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) and GSH adsorbs light at 340 nm more strongly than either GSH or CDNB alone. Substrates and auxiliary enzymes used for enzyme assays were obtained from Sigma Chemical Corp., St. Louis.

Hemoglobin Studies. Hemoglobin spectra were determined in a Beckman Model 25 spectrophotometer. Methemoglobin levels were determined using our modification of the method of Evelyn and Malloy.

Enzyme Purification. GST was purified on a GSH-Sepharose affinity column as described by Simons and Vander Jagt.

Immunologic Studies. Anti-GST antibody was a generous gift from Dr. Yogesh Awasthi (University of Texas, Galveston). The antibody had been raised in goats against the anionic GST of human lung. The dilutions of the antibody in phosphate buffered saline (pH 7.4, 9 parts PBS:1 part 0.10 mol/L potassium phosphate buffer, pH 7.4, 9 parts 0.154 mol/L NaCl) were incubated with purified GST, allowed to stand for two hours at room temperature, and then 1/4 part of Staph A (IgG sorb [The Enzyme Center, Malden, MA]) was added. After mixing on a tube rotor for an additional 30 minutes the tubes were
centrifuged and the supernatant removed and assayed for GST activity.

RESULTS

Erythrocyte GST levels in patients and controls. The levels of red cell GST in the patient group and normal values are presented as a function of the age-related enzyme hexokinase in Fig 1. It is apparent from these data that there was a significant correlation ($P < .001$), with the activity of hexokinase indicating that GST is affected by red cell age.

Of the 513 patient samples assayed, two were distinctly and repeatedly abnormal. One of these represented a child with glutathione deficiency due to glutathione synthetase (GSH-S) deficiency. A sibling manifested the same constellation of findings, i.e., very low red cell GSH, very low GSH-S, and moderately lowered GST levels, while the parents had half-normal GSH-S levels with normal levels of GSH and GST. The results in this family were recently published in detail; we concluded that the low GST level was secondary to the loss of GSH, which normally stabilizes the enzyme.

The second patient with red cell GST deficiency presented quite a different picture. As indicated below, his red cell GSH levels were quite normal. He is designated by his initials, TF, and more detailed clinical and experimental data on this subject are presented below.

Clinical findings in patient TF. TF is a 25-year-old white man, a US Air Force Officer who experienced no significant medical problems until June 1985, at which time he developed a febrile illness associated with mild hemolytic anemia. Hemoglobin levels ranged between 12.7 and 13.1 g/dL. Erythrocyte indices, morphology, serum iron and ferritin and hemoglobin electrophoresis were all normal. Records do not reveal whether splenomegaly was present. In December 1985 he again developed a mild hemolytic anemia in association with infectious mononucleosis. During this period he was noted to have splenomegaly, documented by liver/spleen scan, and his hemoglobin concentration ranged from 12.5 to 12.9 g/dL.

Since January 1986 the patient has remained asymptomatic. Splenomegaly documented by repeat liver/spleen scan has persisted. He continues to have well-compensated mild hemolysis manifested by uncorrected reticulocyte counts ranging from 2.0% to 3.8% and low haptoglobin levels. The WBC count has ranged from 2,500 to 4,500 per μL, with occasional mild lymphocytopenia; and the hemoglobin level, hematocrit, and platelet counts have remained within normal range. Hepatic enzyme levels have been normal, although total bilirubin levels have ranged from 2.4 to 3.9 mg/dL, with a predominance of indirect reacting bilirubin. Abdominal ultrasound revealed cholelithiasis, and an uneventful cholecystectomy was performed in March 1987. Further routine studies to identify the cause of hemolysis, including Coombs test, osmotic fragility testing, hemoglobin electrophoresis at alkaline and acid pH, and Heinz Body preparation with and without oxidant, were all normal. The patient was adopted early in life and does not know his natural parents. He has no children.

Enzyme assays in patient TF. The results of enzyme assays performed at a time that the patient had nearly completely recovered from a hemolytic episode are presented in Table 1. The decreased GST level was confirmed on two subsequent occasions: first, 2 weeks after the original sample was obtained and then 4 months after the original sample. The GST activities on those occasions was very similar to the original level obtained, at 0.93 and 1.10 U/g hemoglobin (Hb). The enzyme activity of leukocytes obtained from TF was modestly decreased at 94.2 U/g protein (NV = 140.9 ± 24.1 U/g protein [mean ± 1 SD]). Platelet activity was 30.7 U/10^{12} platelets (NV = 55.1 ± 27.0 U/10^{12} platelets). Plasma enzyme activity was 6 U/L in the case of both TF and control. Quadruplicate assays carried out on cultured...
skin fibroblasts from TF revealed 225.87 ± 18.88 U/g protein (NV 293.92 ± 22.11). Of the other assays only the activity of pyrimidine 5' nucleotidase and creatine were increased. These are the expected findings in a patient who has recovered from a recent hemolytic episode, since the activities of most of the age-dependent enzymes with the exception of pyrimidine 5' nucleotidase decline very rapidly after maturation of the reticulocyte.19,20

To determine whether an inhibitor of GST might be present, mixtures of normal hemolysates and hemolysates from TF were assayed for GST activity. The activity found was as calculated, suggesting that no inhibitor was present. A 40% suspension of normal red cells in plasma from TF was incubated for one hour at 37°C. There was no significant change in GST activity.

Stability of erythrocyte GST from normal subjects and TF. Density separation of patient and control red cells on Percoll-Histopaque yielded reticulocyte-rich and reticulocyte-poor fractions. Table 2 presents data regarding GST activity in normal subjects and patient TF. We found that the ratio of activity of reticulocyte-rich to reticulocyte-poor fractions was between 2:1 and 3:1 in all but one of the control samples. The sample from patient TF showed a similar ratio.

Purified enzyme from the patient and a normal control was heated to 42°C in a 10 mmol/L KPO 4 buffer, pH 7.0, containing 0.7 mmol/L beta-mercaptoethanol, and enzyme activity was determined at ten-minute intervals. The normal enzyme activity declined with a T1/2 of approximately 7.5 minutes, and the enzyme from TF with a T1/2 of 5.3 minutes. This difference is probably within the limits of experimental error.

Intraerythrocytic GSTfunction. To determine whether intracellular conjugating activity reflected the decreased GST activity in the hemolysate from the patient, the loss of red cell GSH after addition of CDNB from the cells of TF was compared with that of a normal control. A 33% suspension of four times-washed red cells in PBS was warmed to 37°C, and sufficient CDNB to provide a final concentration of 0.6 mmol/L was added. GSH levels were measured at frequent intervals. The results of such a study are illustrated in Fig 2. The initial rate of decline was much more rapid in the normal cells, more than 50% of the GSH disappearing within the first two minutes compared with only 25% of the GSH from the cells of TF. After the initial rapid decline in GSH levels from the normal cells, the rate slowed so that it was identical to the rate observed in TF cells.

A 50% suspension of washed erythrocytes from TF and a normal control were incubated for one hour in PBS containing 5 mmol/L glucose and 1 or 2 mmol/L CDNB. Samples were removed at intervals, washed in PBS with glucose, lysed in 10 mmol/L phosphate buffer, pH 8.0, and destomated by centrifugation at 15,000 g for ten minutes. The optical spectra in the 590- to 650-nm range and the methemoglobin concentration were measured. There was no difference between the red cells of TF in the amount of methemoglobin formed with 1 mmol/L CDNB or with 2 mmol/L CDNB after 15 minutes, but the methemoglobin formed at one hour with 2 mmol/L CDNB was 9.6% compared with 4.91% in the case of the control. We consider this difference to be of doubtful significance and did not repeat the studies because...
of the constraints introduced by the limited availability of blood samples. There were no other differences between the spectra of TF and control hemolysates.

**Kinetic properties.** The kinetic properties of the enzyme were investigated at 25°C using affinity-purified GST with a specific activity of 5 U/mg protein. Five independent determinations of the $K_m$ for GSH gave a value of 0.317 ± 0.069 mmol/L for normal enzyme and 0.236 ± 0.029 mmol/L for enzyme from TF. The $K_m$ for CDNB was 0.649 ± 0.093 mmol/L for normal enzyme and 0.847 ± 0.178 mmol/L for enzyme from TF.

**Immunologic studies.** Figure 3 shows the relationship between the amount of antibody added to purified enzyme and the percentage of the enzyme removed. It is apparent that the antigenicity of the enzyme from TF and from the normal control was the same.

**DISCUSSION**

Among 513 patients with various hematologic disorders, the investigators found one patient with markedly diminished GST activity that could not be explained, except as an inherited enzyme deficiency. The enzyme activity of the patient’s mixed RBCs was approximately 15% of the mean normal value. No deficiency of erythrocyte GST transferase has been described previously, except for the cases of deficiency secondary to glutathione synthetase deficiency that we documented recently.14 Not even electrophoretic variation of the enzyme has been detected previously, although a kinetically atypical enzyme was recently found in one of 100 individuals whose RBC enzyme was surveyed.15 A null allele of the liver GST1 locus has been reported, but since the liver also expresses the closely related GST2 locus, only a modest 35% reduction of enzyme activity was observed and no clinical consequences were documented.12

The residual enzyme of our patient did not show any major kinetic abnormality. The minor differences found are probably within the limits of experimental variation. The patient’s GST was normally stable to heating, or very nearly so. Moreover, comparison of a reticulocyte-rich and reticulocyte-poor fraction of the patient’s red cells suggested that the enzyme was stable in vivo. In any case, in interpreting the results of characterization of the residual enzyme one must take into account the possibility that more than one genetically determined form of GST may exist in erythrocytes; what might be taken for a structurally abnormal residual enzyme could be a minor form of the enzyme that is normally present but its properties obscured by the large amounts of the major form that are present. Such a minor form has been described but is said to represent less than 5% of the total enzyme in normal cells.21 It is quite likely that the small amount of enzyme activity that could not be sedimented with antibody against lung GST may represent this enzyme.

It is difficult to be certain whether the relatively severe deficiency of the enzyme in RBCs was functionally significant. When incubated with CDNB the initial rate of loss of GSH from the cells of TF appeared to be less rapid than the loss of GSH from control cells. Interpretation of the results of such experiments is complicated by the relatively high nonenzymatic rate of GSH-CDNB conjugation, which cannot be controlled in this type of study. Moreover, the latter portion of the reaction rate may be influenced by the rapid inactivation of GST by CDNB in the absence of high concentrations of GSH.

It is attractive to suggest that a cause-and-effect relationship exists between TF’s GST deficiency and his anemia. However, it is important to bear in mind the fact that the patient’s RBCs were examined enzymatically precisely because he had experienced episodes of hemolytic anemia and continuing low levels of hemolysis. Because it is patients with hemolytic disease whose RBCs are most commonly subjected to biochemical study, the association of deficiencies with hemolytic disease is predictable regardless of whether the enzyme deficiency causes the disorder. There are a number of RBC enzyme deficiencies that do not have any clinical consequences,24,25 and here family studies can be decisive. The complete lack of any known relatives of this patient deprives us of the opportunity to rigorously test the question of whether GST deficiency causes hemolytic anemia. It is tempting to speculate that when RBCs are unable

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**Fig 2.** The effect of 0.6 mmol/L CDNB on the GSH levels of RBCs from patient TF and normal control cells. The initial rate of disappearance of GSH from the cells of TF was less rapid than the loss of GSH from the control cells. 0 T.F.—no CDNB; △ Control—no CDNB; ◆ T.F.— + CDNB; ▲ Control— + CDNB.

**Fig 3.** The relationship between the amount of antibody added to purified enzyme and the percentage of the enzyme removed. It is apparent that the antigenicity of the enzyme from TF and from the normal control was the same.

**DISCUSSION**

Among 513 patients with various hematologic disorders, the investigators found one patient with markedly diminished GST activity that could not be explained, except as an inherited enzyme deficiency. The enzyme activity of the patient’s mixed RBCs was approximately 15% of the mean normal value. No deficiency of erythrocyte GST transferase has been described previously, except for the cases of deficiency secondary to glutathione synthetase deficiency that we documented recently.14 Not even electrophoretic variation of the enzyme has been detected previously, although a kinetically atypical enzyme was recently found in one of 100 individuals whose RBC enzyme was surveyed.15 A null allele of the liver GST1 locus has been reported, but since the liver also expresses the closely related GST2 locus, only a modest 35% reduction of enzyme activity was observed and no clinical consequences were documented.12

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to conjugate certain toxic substances they encounter in the circulation that the cell is damaged and its life span is shortened. Our preliminary attempts to show preferential damage to GST deficient RBCs in vitro were disappointing in this respect but can hardly be considered to be conclusive, since there are many different substances that can be detoxified by GST and since the choice of CDNB as a model substance is quite arbitrary and could yield misleading results.

We had earlier suggested\(^\text{12}\) that GST might function in the erythroblast as a heme transport protein. If that were so, then a severe deficiency of the enzyme might lead to impaired hemoglobin synthesis and a thalassemia-like clinical state. This did not occur in the investigators' patient, and it seems unlikely that this is an important physiologic function of GST.

Now that we know that GST deficiency does indeed exist, screening a larger population of normal subjects for this enzyme deficiency and searching for other patients with hemolytic deficiency who lack GST should help to determine whether this deficiency causes a disease state or whether it is another nondisease of the erythrocyte.

**REFERENCES**

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