Phosphoglycolate Phosphatase and 2,3-Diphosphoglycerate in Red Cells of Normal and Anemic Subjects

By Rosa Somoza and Ernest Beutler

Red cell phosphoglycolate phosphatase (PGP) and 2,3-diphosphoglycerate (2,3-DPG) were investigated in normal and anemic patients and rabbits. In hemolytic anemia and blood-loss anemia, characterized by a young red cell population, there was an increase in both phosphoglycolate phosphatase activity and 2,3-diphosphoglycerate levels. In aplastic anemia, the phosphoglycolate phosphatase activity was normal, but the 2,3-diphosphoglycerate values were nonetheless increased. Thus, no relationship was found between phosphoglycolate phosphatase activity and 2,3-diphosphoglycerate levels. The lack of correlation between the activity of phosphoglycolate phosphatase and 2,3-DPG levels suggests that modulation of phosphoglycolate phosphatase activity does not control the level of 2,3-DPG in erythrocytes.

MATERIALS AND METHODS

Reagents and enzymes were obtained from Sigma Chemical Company, St. Louis, MO. Orthophosphoric acid $^{32}$P was obtained from New England Nuclear Corporation, Boston, MA.

A total of 35 human subjects were studied. Included were 19 normal adults, 12 patients with various types of hemolytic anemia, and 4 patients with hypoplastic anemias. One of the latter had Diamond-Blackfan’s syndrome and had not been transfused. The other three, one with pure red cell aplasia, one with aplastic anemia, and one with a refractory sideroblastic anemia, had been extensively transfused.

An animal model, consisting of rabbits with various forms of experimentally induced anemia, was also investigated. Anemia was induced as follows.

1. Mustargen injection (nitrogen mustard) of 2 mg/kg of body weight intravenously to produce moderate anemia with reticulocytopenia.
2. Drawing 20 ml of blood/day for 5 days to produce severe anemia with a young red cell population.
3. A combination of nitrogen mustard treatment (1, above) and bleeding (2, above) to produce severe anemia.
4. Injection of phenylhydrazine, 30 mg subcutaneously, for 3 days to produce moderate hemolytic anemia.

Hemoglobin determinations, white cell, and reticulocyte counts were performed according to standard techniques. 2,3-Diphosphoglycerate levels were estimated spectrophotometrically by using a modification of the method described by Keitt. Hexokinase was assayed spectrophotometrically.

Phosphoglycolate phosphatase activity was measured by incubating hemolysates with radioactive phosphoglycolate, forming a phosphomolybdate complex with the $^{32}$P, released from the substrate and extracting the complex into isobutanol-benzene. Hemolysates were prepared after filtering blood collected in 1 mg EDTA/ml of blood through a microcrystalline cellulose alpha-cellulose column to remove platelets and leukocytes. The erythrocytes were washed twice in 0.154 M NaCl and were lysed by freezing and thawing in water. $^{32}$P-Phosphoglycolate was synthesized according to a previously described method. The assay mixture contained 2 M Bis-Tris, pH 6.5, 20 μl; 0.1 M MgCl$_2$, 5 μl; 1 M KCl, 20 μl; 0.4 M phosphoglycolate, 2 μl; 50-500 μCi $^{32}$P-glycolate; and H$_2$O to bring volume to 150 μl. The assay was started by adding the enzyme in a volume of 50 μl, and the mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 100 μl of 10% trichloracetic acid (TCA). A blank system was prepared by adding TCA prior to the addition of the enzyme source. After vortexing, the mixture was centrifuged at 1,500 g for 10 min at 4°C. Two-tenths milliliter of the extract was added to a mixture of 0.1 ml 4N H$_2$SO$_4$, 0.1 ml 5% ammonium molybdate, and 0.6 ml of water. After mixing well, 2 ml of a 1:1 isobutanol-benzene solution was added. After vortexing for 30-40 sec, the phases were allowed to separate and 1...
ml of the upper phase was pipetted into a 10-ml glass counting vial. 

$^{32}$P activity was estimated using the Cerenkov effect. One unit of enzyme activity represents the amount of enzyme that releases 1 

RESULTS

In 19 normals, the phosphoglycolate phosphatase (PGP) activity was $1.23 \pm 0.10$ IU (mean $\pm$ 1 SD)/g hemoglobin, and the level of 2,3-diphosphoglycerate was $11.87 \pm 1.6$ mmole/g hemoglobin (Hb). In the 9 women, the mean PGP activity was $1.19 \pm 0.106$ IU/g Hb, while in the 10 men, the activity was $1.27 \pm 0.093$ IU/g Hb. The mean 2,3-DPG level of the 9 women was $12.68 \pm 1.43$ mmole/g Hb, while that in the men was $11.13 \pm 1.43$ mmole/g Hb. Although this difference is statistically significant, it is sufficiently small so that in subsequent analysis, the results of men and women have been calculated together. In normal rabbits, the mean phosphoglycolate phosphatase activity was $2.74 \pm 0.24$ IU/g Hb, and the level of 2,3-diphosphoglycerate was $17.54 \pm 0.92$ mmole/g Hb.

As expected, patients with hemolytic anemia manifested increased activity of red cell hexokinase, a good marker of red cell age. As shown in Fig. 1, there was a positive correlation between hexokinase activity and PGP activity in these patients ($r = 0.6; t = 2.46$). This finding confirms our previous observation$^{10}$ that density-separated young red cells have higher PGP activity than do older cells. The level of PGP in all of the patients with hemolytic anemia was increased, and that of 2,3-DPG was increased the most (Fig. 2). The four patients with aplastic anemia also manifested an increase in 2,3-diphosphoglycerate levels, but in contrast to the patients with hemolytic anemia, the activity of red cell PGP was normal or nearly so. The relationship of red cell 2,3-DPG to the hemoglobin level of the blood is shown in Fig. 3.

Because the red cells in patients with aplastic anemia were largely those that had been transfused, further investigations of the effect of anemia with and without reticulocytosis were carried out in rabbits. As shown in Fig. 4, rabbits, like humans, respond to anemia by elevating red cell 2,3-DPG levels. Like humans, too, rabbits with elevated reticulocyte counts manifested an increase in red cell PGP activity. However, rabbits in which the reticulocyte response to
anemia had been prevented by treatment with nitrogen mustard manifested an identical elevation of 2,3-DPG levels as did those with a reticulocytosis; the activity of PGP in the red cells of these marrow-suppressed animals was normal. It was clear from these studies that the 2,3-DPG response was independent of the age of the red cell population and of the level of PGP in the erythrocytes.

**DISCUSSION**

Anemia is known to be associated with a decreased affinity of hemoglobin for oxygen and this is related to increased levels of red cell 2,3-diphosphoglycerate. The regulation of red cell 2,3-diphosphoglycerate content has thus come to be recognized as an important adaptive mechanism to combat tissue hypoxia. One of the factors that normally regulates the level of 2,3-diphosphoglycerate in red cells is the activity of diphosphoglycerate phosphatase (DPGP). Small amounts of phosphoglycolate have been identified in red cells. Since the activity of DPGP is strongly stimulated by phosphoglycolate, and PGP is the enzyme that degrades this compound, a relationship might exist between the 2,3-diphosphoglycerate level and PGP activity (Fig. 5). In earlier studies, we found no relationship between the PGP activity of normal persons and the level of their red cell 2,3-DPG. However, a relationship between PGP in various species and their red cell 2,3-DPG level was documented. Analysis of our present data demonstrated that there was no significant correlation between red cell 2,3-diphosphoglycerate content and PGP activity in anemic patients. In aplastic anemia, whether in patients or in experimental animals, the levels of 2,3-diphosphoglycerate were significantly increased despite normal values of phosphoglycolate phosphatase.

The finding that no difference in 2,3-diphosphoglycerate levels existed in anemic patients with young red cells and in those with aged cells suggests that the capacity of erythrocytes to respond to the stress of anemia is essentially independent of their age. These findings are in contrast to our earlier findings and
those of Graziano et al.,21 which might suggest that young red cells may be better able to respond to hypoxia by elevating their 2,3-DPG levels.

Our results suggest that mechanisms other than modulation of phosphoglycolate levels of PGP exist to account for the increment of 2,3-diphosphoglycerate in anemia. It is possible that the formation of phosphoglycolate is the regulated step or that modulation of the 2,3-DPG mutase–phosphatase system occurs through some means other than the level of phosphoglycolate.

REFERENCES

Phosphoglycolate phosphatase and 2,3-diphosphoglycerate in red cells of normal and anemic subjects

R Somoza and E Beutler