In Vivo Aging of Transfused Erythrocytes and 2,3-Diphosphoglycerate Levels

By Joseph D. Dickerman, Enrique M. Ostrea, Jr., and William H. Zinkham

Levels of 2,3-diphosphoglycerate (2,3-DPG) and oxygen affinity were measured in a patient with refractory congenital hypoplastic anemia in order to determine the response of aging transfused erythrocytes to a decrease in tissue oxygen tension. Ferrokinetic studies revealed that the rate of erythropoiesis after transfusion was approximately 10% of normal. In the interval between transfusions, the level of 2,3-DPG and P50 rose to values considerably above the normal range as the level of the hematocrit decreased. On five separate occasions, the 2,3-DPG concentration was elevated (average 25.5 μmoles/g of hemoglobin) and the P50 level increased (average 33.2 mm of mercury) 24 hr prior to transfusion. It is concluded that in vivo aging of transfused erythrocytes does not prevent these cells from responding to an increasingly hypoxic environment by elevating 2,3-DPG levels and by decreasing oxygen affinity.

A N IMPORTANT intraerythrocytic adaptation to anemia is a reduction of the affinity of hemoglobin for oxygen. Anaerobic glycolysis, the major pathway of glucose catabolism in the mature red cell, generates a variety of organic phosphates. One of these, 2,3-diphosphoglycerate (2,3-DPG), decreases the oxygen affinity of purified hemoglobin for oxygen.\(^1,2\) In a variety of physiologic and pathologic conditions, an inverse relationship exists between 2,3-DPG levels and the oxygen affinity of intact red cells.\(^3-5\) These observations indicate that alterations in erythrocyte metabolism can affect the ability of the red cell to deliver oxygen to the tissues.

Very little information exists concerning the metabolic capacity of old erythrocytes to respond to changes in tissue oxygen tensions by modulating 2,3-DPG levels. The activity of many red cell enzymes decreases during red cell aging.\(^6-8\) Moreover, old cells have a higher oxygen affinity than young cells.\(^9-11\) Whether these changes in oxygen affinity during cell aging are directly related to alterations in 2,3-DPG levels is unclear. Some investigators have demonstrated elevated levels of 2,3-DPG in young red cells and reduced levels in old cells,\(^7,11,12\) while others have reported normal 2,3-DPG/g of hemoglobin in young cells.\(^10,13\) Whatever mechanisms are responsible for the increasing
affinity of red cells for oxygen during aging, one would predict that in vivo aging of transfused erythrocytes would impair the ability of these cells to oxygenate tissues.

Evidence to the contrary was presented by Edwards et al., who showed that the oxygen dissociation curve was shifted to the right 15 days after transfusion in a patient with severe aplastic anemia. More recently, Opalinski and Beutler reported that 2,3-DPG levels in red cells obtained from three patients with aplastic anemia 1 mo after transfusion were slightly but significantly above the normal mean.

A patient with refractory, congenital hypoplastic anemia requiring transfusions every 6–8 wk presented an opportunity to study the effects of in vivo cell aging on 2,3-DPG levels and oxygen transport. Serial determination of 2,3-DPG concentrations and oxygen dissociation curves revealed a continuing and progressive increase of 2,3-DPG and P50 levels during the interval between transfusions. Thus, in vivo aging of transfused erythrocytes does not prevent these cells from responding to an increasingly hypoxic environment by elevating 2,3-DPG levels and by decreasing oxygen affinity.

CASE REPORT

K.C. (JHH No. 86 73 08) is a 15-yr-old white female who has severe, refractory congenital hypoplastic anemia requiring 2 U of packed red blood cells every 50–70 days. The patient takes no medication or other chemical substance known to affect erythropoiesis. She is far below the third percentile in height (110 cm) and weight (24 kg) and has severe, transfusion-induced hemosiderosis. The edge of the liver is palpable 10 cm below the costal margin in the right nipple line, and the degree of enlargement does not vary with the hemoglobin level. The white blood and platelet counts are slightly decreased. The reticulocyte count varies between 0% and 0.1% and has never been greater than 0.1%, even on the day before transfusion. Bone marrow aspiration, just prior to the study period, revealed a myeloid to erythroid ratio of 26:1, lymphocytosis, hemosiderin-laden reticulum cells, and no siderocytes. The level of serum haptoglobin was 1/512 (normal 1/64–1/512), direct and indirect Coombs’ tests were negative, the total iron-binding capacity was 232 μg/100 ml with a serum iron of 232 μg/100 ml. The urinalysis was normal except for a positive Prussian blue reaction. In spite of many transfusions, there were no demonstrable red cell antibodies in the patient’s serum. The serum phosphorus was 4.5 mg/100 ml. The pH of the blood just prior to one of the transfusions was 7.40 and 16 hr after the transfusion was 7.41. Roentgenogram of the chest was normal.

MATERIALS AND METHODS

In addition to the patient cited above, 83 subjects were studied: 52 normal, young white adults; 12 patients with iron deficiency anemia; 12 patients with sickle cell anemia; three patients with red cell pyruvate kinase deficiency; and four patients with hereditary spherocytosis. Venous blood collected in ethylenediaminetetraacetic acid (EDTA) or heparin was used. Hemoglobin was estimated by the cyanmethemoglobin method, and microhematocrits and reticulocytes were measured by standard techniques. 2,3-DPG levels were determined on a Beckman Model DU spectrophotometer using Beutler’s modification of Krimsky’s technique. The 2,3-DPG standard was obtained from the Sigma Chemical Company as the ditris salt, and the enolase and phosphoglyceromutase were obtained from the Boehringer Mannheim Corporation. Serum iron and total iron-binding capacity were measured by the Bio-Science Laboratories in Van Nuys, Calif. The haptoglobin was quantified by an immunoprecipitin technique (unpublished method developed by Dr. Priscilla Gilman) using goat antiserum to human α2-haptoglobin obtained from Hyland, Travenol Laboratories (Costa Mesa, Calif.). Blood for transfusion was obtained from adult volunteers, with 450
AGING OF TRANSFUSED ERYTHROCYTES

Fig. 1. Relationship of 2,3-DPG and P50 to hematocrit in patient with congenital hypoplastic anemia. Cross-hatched areas represent range of normal values. PRBC represents packed red blood cells.

ml of whole blood added to 67.5 ml acid-citrate-dextrose (National Institutes of Health, Formula A). The red cells were washed two times with 250 ml of nonbuffered 0.14 M sodium chloride and were immediately infused into the patient.

A semimicromethod was used to determine the oxygen dissociation curve. Eight-tenths to 1.0 ml of blood was equilibrated in the tonometers of the Astrup blood gas machine with a humidified O2-N2-CO2 gas mixture for 10–15 min at varying concentrations of O2 and N2 and a fixed CO2 concentration. The pH of the gassed blood was determined by a radiometer capillary pH glass electrode, and the PO2 was measured by a Clark-type microelectrode. Oxygen saturation was spectrophotometrically determined by the method of Dubowski.9 The Bohr factor2 was used to correct each PO2 reading from the original plasma pH to a pH of 7.4. The oxygen dissociation line was drawn by plotting log S02/100 vs. log PO2. A minimum of three to four points were determined for each curve. The PO2 at which 50% of the blood was saturated with oxygen (P50) was read directly from the graph.

Assessment of red cell production was accomplished by measuring the incorporation of 59Fe into red blood cells.16 Plasma iron clearance was estimated using 59Fe.16 Three microcuries of 59Fe were incubated with ABO-Rh-compatible plasma for 30 min and were then injected into the patient. The blood volume was measured by the 51Cr technique.16

RESULTS

Results of the determination of 2,3-DPG levels on normal individuals and patients with various types of anemia are listed in Table 1. These data are presented to show the range of 2,3-DPG concentrations obtained in this laboratory. A statistically significant difference was noted between males and females, p <0.02. This difference has been observed by some investigators21 but not by others.22,23 Values for 2,3-DPG concentrations in patients with iron deficiency anemia, sickle cell anemia, red cell pyruvate kinase deficiency, and hereditary spherocytosis agree with results previously reported.24–28 In patients with red cell pyruvate kinase deficiency, the level of 2,3-DPG was proportional to the reticulocyte count and was inversely proportional to the
Table 1. 2,3-DPG, Hemoglobin, and Reticulocyte Measurements in Normal White Adults and Patients With Various Types of Anemia

<table>
<thead>
<tr>
<th>Subject</th>
<th>2,3-DPG (µmoles/gHb)</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Reticulocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adult males and females</td>
<td>15.2 ± 1.5*</td>
<td>15.67 ± .65†</td>
<td>—</td>
</tr>
<tr>
<td>Normal adult males</td>
<td>14.7 ± 1.2†</td>
<td>15.67 ± .65†</td>
<td>—</td>
</tr>
<tr>
<td>Normal adult females</td>
<td>15.7 ± 1.63†</td>
<td>13.97 ± .91†</td>
<td>—</td>
</tr>
<tr>
<td>Patients with nutritional iron deficiency anemia</td>
<td>24.54 ± 3.87</td>
<td>7.73 ± 1.17</td>
<td>—</td>
</tr>
<tr>
<td>Patients with sickle cell disease (hemoglobin SS)</td>
<td>23.16 ± 4.09</td>
<td>7.39 ± 1.21</td>
<td>—</td>
</tr>
<tr>
<td>Patients with red cell pyruvate kinase deficiency</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Patient No. 1 (female)</td>
<td>50.0</td>
<td>6.7</td>
<td>58</td>
</tr>
<tr>
<td>Patient No. 2 (female)</td>
<td>33.5</td>
<td>8.9</td>
<td>33.5</td>
</tr>
<tr>
<td>Patient No. 3 (male)</td>
<td>28.2</td>
<td>9.8</td>
<td>18.7</td>
</tr>
<tr>
<td>Patients with hereditary spherocytosis</td>
<td>13.6</td>
<td>12.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Patient No. 1 (female)</td>
<td>15.4</td>
<td>12.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Patient No. 2 (male)</td>
<td>15.4</td>
<td>9.8</td>
<td>11.7-17.5</td>
</tr>
<tr>
<td>Patient No. 3 (male)</td>
<td>16.4</td>
<td>13.2</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD.
†p <0.02.
‡p <0.001.

hemoglobin concentration. Patients with hereditary spherocytosis exhibited normal levels of 2,3-DPG in spite of an elevated reticulocyte count and normal or slightly decreased hemoglobin concentrations.

The blood used for transfusion in the present study had been stored in acid-citrate-dextrose (ACD) for 11 days and consisted of 525 cc of washed, packed red blood cells containing 111 g of hemoglobin. The reticulocyte count was 1.2%; the 2,3-DPG concentration was 0.38 µmoles/g of hemoglobin, and the P₅₀ was 15.8 mm of mercury. The circulating hemoglobin mass in the patient was approximately 75 g.

In Fig. 1, the first points are values obtained immediately before transfusion. While the transfusion was in progress, 2,3-DPG values decreased to normal. The P₅₀ returned to a normal level 12 hr after transfusion. During the next 56 days, the levels of 2,3-DPG and P₅₀ rose as the level of the hematocrit decreased. Table 2 shows that on five separate occasions the concentration of

Table 2. Values for 2,3-DPG, P₅₀, and Hematocrit 24 Hr Prior to Transfusion

<table>
<thead>
<tr>
<th>Date</th>
<th>P₅₀ (mm Hg)</th>
<th>2,3-DPG (µmoles/g Hb)</th>
<th>HCT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sept. 22, 1971</td>
<td>37.8</td>
<td>25.7</td>
<td>15.9</td>
</tr>
<tr>
<td>Dec. 2, 1971</td>
<td>33.7</td>
<td>23.7</td>
<td>14.9</td>
</tr>
<tr>
<td>Jan. 25, 1972</td>
<td>35</td>
<td>26.9</td>
<td>18.8</td>
</tr>
<tr>
<td>March 20, 1972</td>
<td>29.5</td>
<td>27.9</td>
<td>16.7</td>
</tr>
<tr>
<td>May 3, 1972</td>
<td>30</td>
<td>23.3</td>
<td>15.2</td>
</tr>
</tbody>
</table>
AGING OF TRANSFUSED ERYTHROCYTES

2,3-DPG and $P_{50}$ value were elevated 24 hr prior to transfusion. Ferrokinetic studies revealed that the $t_{1/2}$ of the initial $^{59}$Fe plasma clearance was 7 hr and 45 min. Normal values range from 60 to 120 min. On days 1, 2, 5, 7, 8, 12, 14, and 16 after the transfusion, the per cent incorporation of $^{59}$Fe into the patient's red blood cells was 0.05, 2.8, 3, 4.5, 6, 6.5, 6.8, and 7.5%, respectively. Normal individuals show 60%–80% incorporation of the isotope in 7–10 days. The estimated total blood volume 3 days after transfusion was 1287 cc or 54 cc/kg.

DISCUSSION

The majority of previous investigations concerning the relationship between 2,3-DPG levels, oxygen affinity, and red blood cell age have been done in vitro on stored blood or blood from normal individuals fractionated into young and old populations. There is general agreement that young erythrocytes have a lower affinity for oxygen than old erythrocytes. Less clear is the extent to which these age-related changes in oxygen affinity are due to variations in 2,3-DPG levels. Three studies showed increased levels of 2,3-DPG in young cells separated by centrifugation, whereas another study revealed normal levels.

In vivo studies have demonstrated that the concentration of 2,3-DPG in erythrocytes previously stored in ACD returns to slightly above normal levels on the 6th and 11th day post-transfusion. In addition, an elevated $P_{50}$ was observed in one patient with aplastic anemia transfused 15 days prior to investigation, and 2,3-DPG levels were slightly but significantly elevated in three other patients with the same condition studied 1 mo after transfusion. What has not been demonstrated previously is the supernormal levels of 2,3-DPG generated by erythrocytes exposed to an increasingly hypoxic environment while aging in vivo.

The patient in this study is almost completely aplastic with respect to the erythroid series, as demonstrated by the appearance of the bone marrow, reticulocyte count, ferrokinetic studies, and transfusion requirements. The majority of the red blood cells in the patient represent an aging population exposed to increasing degrees of hypoxia. Even so, as the patient becomes more anemic, the levels of 2,3-DPG and $P_{50}$ increase, events that have been confirmed on five separate occasions. Thus, erythrocytes, aging in vivo, are capable of responding to increasingly hypoxic conditions by elevating concentrations of 2,3-DPG and decreasing oxygen affinity.

An important question to answer is whether the number of red cells produced by the patient could account for the elevation of the 2,3-DPG and $P_{50}$. The reticulocyte count on many occasions varied between 0% and 0.1% on the day before transfusion. Ferrokinetic studies performed during a period of 17 days following one of the transfusions revealed a 90% reduction in the rate of erythropoiesis. Even though ferrokinetic data are not available just prior to transfusion, the persistent reticulocytopenia and the frequency of the transfusion requirements suggest that most of the patient's red cells are provided by transfusions.

To illustrate the capacity of the infused erythrocytes to generate 2,3-DPG,
let us assume arbitrarily that the capacity of the patient to make new red cells is 50% of normal, an estimate that is undoubtedly too high. In previous studies, the level of 2,3-DPG in young cells separated by centrifugation was found to be 17.1 and 17.2 \( \mu \)moles/g of hemoglobin and in old cells was 12.3 and 7.3 \( \mu \)moles/g of hemoglobin. Just prior to transfusion, the patient’s total hemoglobin was approximately 75 g. If one assumes that 50% of the hemoglobin was derived from the patient’s own cells capable of generating amounts of 2,3-DPG similar to those observed in young cells and that the remainder was derived from transfused cells with 2,3-DPG levels similar to those observed in old cells, then the total amount of 2,3-DPG would be 1010 \( \mu \)moles/75 g of hemoglobin or 13.4 \( \mu \)moles/g of hemoglobin. A value of 13.4 \( \mu \)moles of 2,3-DPG/g of hemoglobin is much lower than those exhibited by this patient just prior to transfusion.

Patients with hemolytic disorders characterized by anemia and reticulocytosis have a moderate to marked increase in erythrocyte 2,3-DPG levels. In aplastic anemia and hypoxic states with or without polycythemia, the level of 2,3-DPG is increased and the reticulocyte count is normal or reduced. These observations, together with the results of the present study, suggest that the degree of tissue oxygenation is just as important a determinant of 2,3-DPG levels as cell age.

ACKNOWLEDGMENT

The authors wish to thank Dr. Patricia McIntyre for her help with the ferrokinetic studies and Miss Susan Dixon for her technical assistance.

REFERENCES

13. Hjelm M: The content of 2,3-diphos-
phosphoglycerate and some other phosphocom-
pounds in human erythrocytes from healthy
adults and subjects with different types of
Metcalfe J: Improved oxygen release: Adap-
tion of mature red cells to hypoxia. J Clin
Invest 47:1851, 1968
15. Opalinski A, Beutler E: Creatine, 2,3-
diphosphoglycerate and anemia. N Engl J
Med 285:483, 1971
16. Dacie JV, Lewis SM: Practical Haema-
tology (ed 4), New York, Grune & Stratton,
1968, p 30
17. Beutler E, Meul A, Wood LA: Deple-
tion and regeneration of 2,3-diphosphogly-
ceric acid in stored red blood cells. Trans-
fusion 9:109, 1969
18. Krimsky I: D-2,3-diphosphoglycerate,
in Bergmeyer HU (ed): Methods of Enzy-
matic Analysis. New York, Academic, 1965,
p 238
19. Dubowski KM: Spectrophotometric
determination of blood O2 saturation in in-
fancy, in Sunderland FW, Sunderland FW Jr
(eds): The Clinical Pathology of Infancy.
Springfield, Ill., Thomas, 1967, p 433
20. Severinghaus JW: Blood gas calcula-
21. Woodson RD, Torrance JD, Shappell
SD, Lenfant C: The effect of cardiac disease
on hemoglobin-oxygen binding. J Clin Invest
49:1349, 1970
22. Eaton JW, Brewer GJ: The relationship
between red cell 2,3-diphosphoglycerate and
levels of hemoglobin in the human. Proc
Natl Acad Sci USA 61:756, 1968
23. Hellerstein S, Grady H, Grisolia S:
Normal values of red cell 2,3-diphosphogly-
cerate by a simplified automated method.
J Lab Clin Med 76:171, 1970
24. Hjelm M: Erythrocyte glycolytic inter-
mediates and cofactors correlated with the
hemoglobin concentration in human neo-
nates and adults, in Brewer GJ (ed): Red
Cell Metabolism and Function. New York,
London, Plenum, 1970, p 83
25. Charache S, Grisola S, Fiedler A,
Helleger AE: Effect of 2,3-diphosphoglycerate
on oxygen affinity of blood in sickle cell
26. Keitt AS, Bennett DC: Pyruvate kinase
deficiency and related disorders of red cell
27. Oski FA, Bowman H: A low Km phospho-
olopyruvate mutant in the Amish with reed
cell pyruvate kinase deficiency. Br J
Haematol 17:289, 1969
28. Palek J, Mircevov#{225} L, Brabec V: 2,3-
diphosphoglycerate metabolism in heredi-
tary spherocytosis. Br J Haematol 17:59,
1969
29. Valeri CR, Hirsch NM: Restoration
in vivo of erythrocyte adenosine triphos-
phate, 2,3-diphosphoglycerate, potassium
ion, and sodium ion concentrations follow-
ing the transfusion of acid-citrate-dextrose-
stored human red blood cells. J Lab Clin
Med 73:722, 1969
30. Eaton JW, Brewer GJ, Grover RF: Role
of red cell 2,3-diphosphoglycerate in the
adaptation of man to altitude. J Lab Clin
Med 73:603, 1969
31. Rosenthal A, Mintzer WC, Eisenstein
EB, Nathan DG, Nelson NM, Nadan AS:
The role of red blood cell organic phospho-
phates in adaptation to congenital heart
32. Lenfant C, Torrance J, English E, Finch
CA, Reynafarje C, Ramos J, Faura J: Effect
of altitude on oxygen binding by hemoglo-
bin and on organic phosphate levels. J Clin
Invest 47:2652, 1968
In Vivo Aging of Transfused Erythrocytes and 2,3-Diphosphoglycerate Levels

Joseph D. Dickerman, Enrique M. Ostrea, Jr. and William H. Zinkham