The Effect of Hemoglobin Carbamylation on the Survival of Human Sickle Cell Erythrocytes in Rats

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The im vitro incubation of human sickle cell erythrocytes (SS RBCs) with sodium cyanate results in significant prolongation of their $^{51}$Cr intravascular survival (1, 2). This effect appears to be mediated through carbamylation of the amino terminal valine residue in the hemoglobin S molecule which results in inhibition of erythrocyte sickling (3). Initial clinical studies have shown that the oral administration of sodium cyanate to patients with homozygous sickle cell disease also increases erythrocyte survival and leads to improvement in anemia (4, 5). Sickle cell crises, however, continue to occur in the cyanate treated patients (5). This observation raises serious questions regarding the effectiveness of cyanate treatment in the prevention of localized intravascular sickling, and in the overall clinical management of patients with sickle cell disease.

Pretreatment of rats with (a) ethyl palmitate (EP) which blocks the reticuloendothelial system and produces acute splenic necrosis, and (b) cobra venom factor (CVF) which inhibits the third component of complement has resulted in the development of an animal system suitable for study of the intravascular survival of human erythrocytes (6). It now has been shown that two important similarities exist between the behavior of SS RBCs in humans and the behavior of SS RBCs transfused into rats which have been pretreated with EP and CVF (7). The first is that SS RBCs have a significantly shorter $^{51}$Cr life span than that of control RBCs. Secondly, exposure of rats which had been breathing 100% O$_2$ to a hypoxic environment results in the rapid loss from circulation of from 30–60% of the transfused human SS RBCs. These observations suggest that the rat model might be of value in determining the effectiveness of antisickling drugs in the prevention of accelerated SS RBC loss from circulation during hypoxia. The immediate objectives of our experiments were to determine the effect of cyanate treatment of human SS RBCs on their survival in the rat model and, more importantly, the behavior of carbamylated human SS RBCs in the rat during the stress of hypoxia.

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MATERIALS AND METHODS

The blood from 4 patients with homozygous sickle cell anemia was studied. None of the patients had been transfused for at least 2 mo prior to this study. Their ages were 1, 22, 23, and 33 yr. Each patient had from 87.5 to 93.4% S hemoglobin as determined by acrylamide gel electrophoresis and hematocrit values ranging from 20 to 30%.

Four milliliters of venous blood from each patient was added to 1 ml of sterile acid–citrate–dextrose solution and separated into 2 equal portions. Both were centrifuged and the supernatant plasmas were removed. The packed red cells in one tube were made up to the original volume with a solution of sodium cyanate in normal saline so that the final cyanate concentration was 50 mM. The other tube of packed red cells was made up to volume with normal saline instead of cyanate. Oxygen was bubbled through the red cell suspensions for a period of 3–5 min. Sodium $^{51}$Chromate (50 μCi/ml blood) was then added to each sample, and the mixtures were incubated at 37°C for 90 min with frequent swirling. The erythrocyte suspensions were washed three times with normal saline in order to remove unbound chromium and cyanate. Normal saline was added to bring the final hematocrit to 30–33%. The degree of amino terminal valine carbamylation achieved in the cyanated sample was 2.0 cyanate residues per mole of hemoglobin tetramer.

Erythrocytes from 3 control individuals with A hemoglobin (AA RBCs) were processed both with and without cyanate incubation in exactly the same manner as was the blood from the patients with sickle cell disease.

Erythrocyte survival studies were performed in adult, male Sprague-Dawley rats weighing between 150 to 250 g. Each animal was prepared for the transfusion study according to a technique which has been previously described (7). Briefly, the method involves tail vein injection of a suspension of ethyl palmitate in a dose of 3 g/kg approximately 12–24 hr before the red cell survival study. A second tail vein injection with 10 units of CVF was given about 30 min before the study. The $^{51}$Cr tagged human erythrocyte suspension (1 ml/kg of packed cells) was injected in the animals through the dorsal vein of the penis. Fifteen minutes later, and at intervals of 30 min to 1 hr thereafter, 10 μl capillary blood samples were taken from the tip of the tail for radioassay in a well-type scintillation counter. The radioactive counts for each animal were normalized and plotted on semilog paper in order to determine erythrocyte half-time ($T_{1/2}$) survival. The radioactivity from the first sample was defined as the 100% value. A total of 26 rats were used for these experiments.

Each patient study usually involved simultaneous determination of SS erythrocyte survival in 4 rats. Two animals were injected with cyanate treated SS RBCs and the other 2 with SS RBCs that had not been treated with cyanate. One of the 2 rats injected with cyanate treated SS RBCs was left in room air during the entire $^{51}$Cr survival study while the other was placed in a hypoxic environment (10% O$_2$) following a 2 hr period of exposure to 100% O$_2$ as previously described (7). The $^{51}$Cr survival of control animals, i.e., those transfused with SS RBCs not treated with cyanate and those transfused with carbamylated and noncarbamylated AA RBCs, was determined under the same conditions.

5 Pfaltz and Bauer, Inc., Flushing, NY.
6 E. R. Squibb and Sons, New Brunswick, NJ.
7 This determination was performed by Dr. Anthony Cerami.
TABLE 1
Effect of Cyanate Treatment on the \(^{51}\text{Cr}\) Survival Characteristics of SS RBCs Transfused to Rats

| Patient | Untreated SS RBC | Cyanate treated SS RBC |
|---------|------------------|------------------------|
|         | T-\(1/2\) survival | % loss\(^{a}\) | T-\(1/2\) survival | % loss\(^{a}\) |
|         | in hr (room air) | in 10% \(O_2\) | in hr (room air) | in 10% \(O_2\) |
| J.G.    | 4.5              | 45               | 7.5              | 42               |
| S.E.    | 4.0              | not done         | 6.0              | not done         |
| B.T.    | 2.0              | 43               | 9.5              | 32               |
| S.B.    | 7.7              | 32               | 17.0             | 33               |
| Average | 4.5              | 40               | 10.2             | 36               |

\(^{a}\) During the first 60 min of exposure to 10% \(O_2\) in relation to the blood radioactivity present immediately before hypoxia.

In a separate experiment, the erythrocytes from patient S.B. with sickle cell disease were labeled with radioactive cyanate: Twenty-five \(\mu\)Ci potassium \(^{14}\text{C}\) cyanate\(^5\) was added to the nonradioactive sodium cyanate and this mixture was added in the usual proportions to the SS RBCs after removal of the plasma. The subsequent incubation and washing steps were the same as for \(^{51}\text{Cr}\) labeled RBCs, except that 1.5 ml RBCs/kg rat wt were injected. Following transfusion of \(^{14}\text{C}\)-cyanate labeled blood into the rats 20 \(\mu\)l capillary samples were taken from the animals at the usual intervals. The capillary pipettes containing the blood samples were emptied into counting flasks containing 0.2 ml of 30% hydrogen peroxide. Five milliliters of Aquasol\(^6\) solubilizer was added to the flasks and \(^{14}\text{C}\) radioactivity counted in a liquid scintillation counter. The \(^{14}\text{C}\) RBC survival characteristics were compared to those of \(^{51}\text{Cr}\) labeled noncarbamylated RBCs from the same patient.

RESULTS

The effect of \textit{in vitro} cyanate treatment of SS RBCs on their survival in rats is shown in Table 1. Carbamylation of SS RBCs resulted in considerable prolongation of erythrocyte survival for each of the 4 patients tested. The mean T-\(1/2\) erythrocyte survival of the cyanate treated cells was more than double that of the non-carbamylated SS RBCs. The significance level (\(t\) test on the paired samples) was less than 0.1. The T-\(1/2\) survival of the cyanate treated SS RBCs was shifted into the AA RBC T-\(1/2\) survival range (7) in 2 of the 4 studies performed. The \(^{51}\text{Cr}\) survival of erythrocytes from three Hb A control individuals was not prolonged by cyanate treatment. The mean T-\(1/2\) for untreated AA RBCs was 18 hr while that for cyanate treated Hb AA RBCs was 14.6 hr.

Experiments in which the animals were placed in 10% \(O_2\) following an initial period of 100% \(O_2\) administration showed no essential difference between the cyanate treated and untreated SS RBCs: There was rapid loss from circulation of 32–42\% of the cyanate treated SS RBCs in comparison to a 32–45\% loss of the untreated SS RBCs within the first 60 min of exposure to the hypoxic environment (Table 1). A representative example of a single experiment is shown in Fig. 1.

\(^5\) New England Nuclear, Boston, MA.
\(^6\) New England Nuclear, Boston, MA.
Fig. 1. Effects of variations in \( O_2 \) concentration on the \( ^{51} \)Cr survival of cyanate treated and untreated (control) RBSs from a patient with sickle cell anemia. Hypoxia (10% \( O_2 \)) resulted in rapid loss of SS RBCs which could not be prevented by pretreatment with cyanate.

Fig. 2. Comparison of the survival characteristics of carbamylated SS RBCs (\(^{14} \)C cyanate was used as a label) and noncarbamylated \(^{51} \)Cr labeled SS RBCs (control) from the same patient. Exposure of the animals to hypoxia (10% \( O_2 \)) was associated with a similar rapid drop in both \(^{14} \)C and \(^{51} \)Cr counts.

The survival of SS RBCs labeled with \(^{14} \)C cyanate and \(^{51} \)Cr tagged SS RBCs not treated with cyanate is shown in Fig. 2. The initial portion (100% \( O_2 \)) of the survival curve of noncyanated SS RBC (\(^{51} \)Cr curve) was no different than that of the SS RBCs which had been treated with cyanate (\(^{14} \)C curve). Subsequent exposure to 10% \( O_2 \) for 1 hr resulted in a 31% drop in \(^{14} \)C counts and a 44% drop in \(^{51} \)Cr counts. The characteristics of the 2 survival curves in room air during the next 15 hr were similar.

DISCUSSION

The results of the carbamylated SS RBC survival studies in this rat model are similar to those which have been reported for studies performed in human subjects (1, 2, 4, 5). In both species, the in vitro treatment of SS RBCs with cyanate results in improvement of their survival to the extent that the blood \( T^{-\frac{1}{2}} \) values approach
those for untreated AA RBCs. Since the effect of carbamylation is probably limited to the inhibition of sickling, one can conclude that the shortened survival of SS RBCs in the rat in room air probably relates to continuous erythrocyte sickling in areas in the body with low oxygen concentration. This hypothesis is further strengthened by the fact that exposure of the animals to 100% O₂ during an SS RBC transfusion study also improves SS RBC survival (7). The period of observation during which the animals were breathing 100% O₂ was too short (2 hr), however, to accurately compare the effect of hyperoxygenation alone vs cyanate alone on the survival of SS RBCs.

The degree of hemoglobin carbamylation achieved in our studies is well within the range which has been effective in prolonging SS RBC survival in man (1, 2, 5). Partial correction of hemolytic anemia in patients with sickle cell disease has been achieved with maintenance of a minimum of 0.3 residues of cyanate per mole of hemoglobin (4, 5).

Our studies also indicate that the accelerated loss of a substantial proportion of human SS RBCs from the hypoxic animal’s circulation could not be prevented by cyanate. The following explanations can be offered for the failure of cyanate treated SS RBCs to resist the in vivo effects of hypoxia. (a) The loss of blood radioactivity during hypoxia (“hypoxic drop”) could represent selective removal of those SS RBCs which had not been carbamylated despite incubation with cyanate. This explanation seems most unlikely because the survival characteristics of SS RBCs labeled only with ¹⁴C-cyanate were almost identical to those seen with the ⁵¹Cr labeled RBCs from the same patient. (b) The hypoxic drop could have been the result of selective removal of carbamylated but irreversibly sickled cells. [Deoxygenated SS RBCs can be carbamylated in the sickled state and carbamyla-
tion per se does not reverse sickling (3)]. Also this explanation does not seem probable: The hypoxic drop can best be demonstrated immediately after a period of time in which the animals breathed 100% O₂. If the loss of radioactive activity seen during the subsequent period of hypoxia were due to loss of irreversibly sickled cells, one would not expect that a preceding period of hyperoxygenation of the animal would have been needed in order to demonstrate the hypoxic phenomenon. (c) The hypoxic drop could be dependent on other factors which are not directly influenced by HbS carbamylation. Erythrocyte age, content of hemoglobin F in the individual cell, or changes in membrane pliability or membrane loss due to repeated sickle–unsickle cycles may be responsible.

The foregoing considerations and our previous observations (7) strongly suggest that in homozygous sickle cell disease there are at least two populations of erythrocytes in terms of their survival characteristics in the rat model. One population of cells is exquisitely sensitive to changes in O₂ tension so that they accumulate in the blood of animals breathing 100% O₂ and are rapidly removed from circulation following hypoxia. The second population is also abnormal since it has a shortened survival but differs from the first population in that hypoxia does not seem to accelerate their removal from circulation. Cyanate appears to increase the intra-vascular survival of both populations but does not seem to prevent the accelerated removal of the SS RBCs most sensitive to oxygen changes during the initial period of oxygen deprivation.

The overall interpretation of these results is guarded in terms of their extrapolation to man. The rat model is extremely sensitive to sickling (preliminary studies suggest that the survival of RBCs of patients with sickle cell trait is somewhat
shorter than that of AA RBCs) and may actually overemphasize the response of human SS RBCs to hypoxia. On the other hand, if an antisickling agent such as cyanate fails to improve or prevent the accelerated loss of SS RBCs in the rat model during hypoxia, one might seriously question the effectiveness of that agent in other species during a comparable challenge.

The similarities between the results obtained with carbamylated human SS RBCs in the rat model and in man provide some additional evidence for the potential usefulness of this model in the study of sickle cell disease and the efficacy of various therapeutic agents.

SUMMARY

The \(^{51}\)Cr survival characteristics of cyanate-treated erythrocytes from four patients with sickle cell anemia were studied in rats which had been pretreated with ethyl palmitate and a cobra venom factor. Cyanate-treated sickle cells showed significant improvement in their survival characteristics in room air. Carbamylation, however, did not prevent the accelerated loss of sickle cells from the rat's circulation following exposure to hypoxia. In a single experiment \(^{14}\)C-cyanate was used both as an erythrocyte label and carbamylating agent. The drop in \(^{14}\)C activity following exposure to hypoxia was of the same magnitude as that observed with noncarbamylated \(^{51}\)Cr-labeled sickle cells. These findings suggest heterogeneity for human sickle erythrocytes in the rat model in terms of their sensitivity to variations in oxygen concentration. Carbamylation fails to protect that population of cells which is most sensitive to the stress of severe hypoxia. The significance of these findings in relationship to human disease has not been defined, but the similarities between the behavior of cyanate-treated sickle cells in humans and in the rat suggest that this simple animal model may be of considerable value in the study of sickle cell disease and its treatment.

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