Decreased Life Span and Membrane Damage of Carbamylated Erythrocytes In Vitro

By Thomas A. Lane and Edward R. Burka

Red blood cells exposed to cyanate (CNO) in vitro have a concentration-dependent decreased cell survival time associated with an inhibition of the ability of the cell membrane to synthesize lipids. The ½ of rabbit erythrocytes exposed to 30 mM or 50 mM cyanate for 1 hr at 37°C is reduced from the normal 24 days to 15 and 9 days, respectively. The cyanate-induced defect in membrane lipid metabolism is irreversible. Carbamylation of membrane proteins and damage to membrane metabolism are minimized by limiting exposure in vitro to 15 mM cyanate at 4°C for 30 min. Cells carbamylated under these conditions do not have a shortened life span. Levels of globin carbamylation of 0.5 moles CNO/mole hemoglobin, shown to be clinically effective in prolonging the life span of sickle erythrocytes, are obtained under these conditions and reach maximal levels after only 30 min of incubation. Carbamylation of blood in CPD anticoagulant is inferior to either ACD or heparin. The findings indicate that adequate carbamylation of sickle erythrocytes with minimal red cell membrane damage can be achieved without significant modification of the standard plasmapheresis procedure utilized by the working blood bank.

The chemical modification of hemoglobin S represents a new approach to the treatment of sickle cell anemia. Treatment with cyanate (CNO), an agent which inhibits sickling by carbamylating globin chains, has already undergone clinical trials, but long-term oral therapy is associated with unacceptable neurotoxicity, weight loss, and cataracts. Consequently, carbamylation of hemoglobin S by extracorporeal treatment of erythrocytes, avoiding the systemic effects of carbamylation, has gained appeal as an alternative to oral therapy.

Membrane proteins are also extensively carbamylated during in vitro exposure of erythrocytes to cyanate. Evidence that carbamylation alters the red cell membrane is the increased autohemolysis of carbamylated erythrocytes. Since the integrity of the erythrocyte membrane is a major determinant of erythrocyte life span, this alteration may shorten the cell life span. The fact that carbamylated sickle erythrocytes, despite the prevention of sickling, are not restored to a fully normal life span suggests that cyanate may indeed adversely influence erythrocyte survival.

The purpose of this study was twofold: (1) to determine if cyanate adversely affects erythroid cell life span and membrane function, and (2) to determine whether an adequate level of globin carbamylation can be obtained in vitro under conditions that minimize adverse effects on the cell membrane. The re-
Results indicate that exposure of erythrocytes to cyanate in vitro causes a concentration-dependent decrease in cell life span which is associated with an inhibition of the ability of the cell membrane to synthesize lipid.

**MATERIALS AND METHODS**

The methods have been described in detail in earlier publications. Blood obtained from patients with sickle cell anemia and autoimmune hemolytic anemia had a reticulocyte count between 5% and 13%. Blood obtained from phenylhydrazine-treated rabbits had a reticulocyte count greater than 60%. Unless otherwise specified, blood was collected in heparinized tubes, and all procedures were performed at 0°C-4°C.

**Conditions of Incubation**

The incubation mixture, usually 8 ml in total, contained equal amounts of washed packed cells and a complete Krebs-Ringer buffer containing $10^{-4}$ M calcium chloride. Lipid synthesis was determined by adding 1-5 μCi of $^{14}$C-glycerol and incubating with agitation at 37°C as described in the text. In some experiments, cells were preincubated with cyanate ("preincubation phase") before the addition of $^{14}$C-glycerol ("synthetic phase"). Vincristine was used in a final concentration of $1.5 \times 10^{-6}$ M, and puromycin was added to a final concentration of $10^{-5}$ M.

Adequacy of the procedure to remove unreacted intracellular cyanate was evaluated by examination of blood exposed to 30 mM cyanate containing trace amounts of $^{14}$C-cyanate. Samples of whole blood, membrane-free hemolysate, erythrocyte membranes, and protein-free hemolysate were counted both before and after washing the cells three times with phosphate-buffered saline. The protein-free lysate was prepared by precipitating membrane-free hemolysate with a final concentration of 10% trichloroacetic acid. After the washing procedure the membrane-free hemolysate contained less than 0.3% of the initial quantity of unbound cyanate.

Incubations were terminated by the addition of 2 volumes of ice cold phosphate-buffered saline, and the cells were washed twice in the same solution. The washed cells were lysed with 4 volumes of 20 mOsm phosphate buffer, pH 7.4, and the cell membranes were separated by centrifugation at 43,500 g for 10 min and then washed three times with 20 mOsm buffer.

**Analytical Methods**

The washed membranes, suspended in 2 ml of 20 mOsm phosphate buffer, were extracted with 5 ml each of methanol and chloroform. The membrane lipids were analyzed by thin-layer chromatography on silica gel G, silicic acid column chromatography, and partial alkaline hydrolysis.

The degree of carbamylation of hemoglobin in membrane-free hemolysates was kindly determined by Dr. A. Cerami of Rockefeller University, New York, by the valine–hydantoin method.

Hemocytometry, enumeration of reticulocytes, and hematocrit determinations were done by standard methods.

Radioactivity was determined in a liquid scintillation counter and sufficient counts accumulated to reduce the error to less than 3%.

**Erythrocyte Survival**

Erythrocytes were tagged with radiochromate in vitro by adding whole blood to special ACD solution and tagging with 20-50 μCi of $^{51}$Na-chromate for a period of 1 hr at room temperature or 37°C. When cells were carbamylated, they were simultaneously exposed to the indicated concentration of sodium cyanate. Control studies showed that the presence of cyanate did not inhibit the chromation of hemoglobin. The blood-ACD mixture was then injected into the donor rabbit via a marginal ear vein and the standard, representing 100% survival, was obtained 24 hr after injection. Subsequent samples were obtained in 0.5-ml amounts two to three times weekly. The percentage of radioactivity of subsequent samples was calculated from the standard and corrected by the hematocrit value. The mixture of carbamylated cells and ACD, when chromated and kept aseptically for periods up to 4 wk, did not have chromium elution rates greater than those of noncarbamylated cells.
Table 1. Half-Time of Survival of Carbamylated Rabbit Erythrocytes*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Half-Time of Survival (Days)</th>
<th>Range</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>22–26</td>
<td>23.8 ± 1.5t</td>
</tr>
<tr>
<td>15 mM CNO⁻, 4°C</td>
<td></td>
<td>18–24</td>
<td>21.0 ± 2.2t</td>
</tr>
<tr>
<td>30 mM CNO⁻, room temperature</td>
<td></td>
<td>13–17</td>
<td>15.3 ± 1.8</td>
</tr>
<tr>
<td>50 mM CNO⁻, 37°C</td>
<td></td>
<td>6–12</td>
<td>9.3 ± 2.8</td>
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*The erythrocytes were simultaneously exposed to radiochromium and cyanate for 1 hr under the conditions described. The results are the averages of six separate studies.
†The difference between these two figures is not statistically significant.

Materials

All chemicals were reagent grade. Labeled precursors and ¹⁴C-cyanate were obtained from New England Nuclear, Boston, Mass. Precoated thin-layer chromatographic plates were obtained from Brinkmann Instruments, Westbury, N.Y. Vincristine was the standard commercial pharmaceutical. Puromycin dihydrochloride was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Recrystallized sodium cyanate was kindly supplied by Dr. A. Cerami of Rockefeller University, New York. For carbamylation studies, commercial Blood-Packs containing either ACD, CPD, or heparin were used (Fenwal Laboratories, Morton Grove, Ill.).

RESULTS

Survival of Carbamylated Erythrocytes

The ultimate criterion of whether or not CNO causes damage to erythrocytes is cell survival. The average half-time of survival of autologous erythrocytes labeled in vitro with radiochromium in six normal rabbits was 23.8 ± 1.5 days (Table 1). Repetitive studies were done in individual animals with chromated cells that either were not exposed or were exposed to 30 or 50 mM CNO at room temperature or 37°C for a period of 1 hr. When plotted either on a linear
scale (Fig. 1A) or a semilogarithmic scale (Fig. 1B), it was apparent that the life span of erythrocytes exposed to cyanate was reduced in a concentration-dependent manner. The average $t_{1/2}$ of survival was reduced to 15.3 and 9.3 days by exposure to 30 and 50 mM cyanate, respectively (Table 1). Simultaneous exposure to cyanate and chromium neither inhibited the chromation of cells nor increased the elution of chromate from the cells.

**Effect of Cyanate on Lipid Synthesis in Reticulocytes**

In order to determine whether the decreased life span of carbamylated erythrocytes is associated with a defect in membrane metabolism, the effects of exposure to cyanate on membrane lipid metabolism were studied. Exposure of human reticulocytes to cyanate depresses incorporation of $^{14}$C-glycerol into total cell lipids in a concentration-dependent manner (Fig. 2). Cyanate does not affect incorporation of the precursor into the two major classes of erythrocyte membrane lipids equally. Twenty-millimolar cyanate causes a $31\%$ decrease in incorporation of $^{14}$C-glycerol into neutral lipids, but at this concentration there is little effect on incorporation into phospholipids. At greater concentrations of cyanate phospholipid synthesis is also decreased. These results show that the presence of the cyanate ion affects erythroid cell membrane metabolism, as evidenced by inhibition of pathways of lipid synthesis. Figure 3 graphically depicts the relationship between decreased cell survival and inhibition of membrane lipid synthesis.

Cyanate-induced inhibition of lipid synthesis is irreversible. The ability of reticulocytes to incorporate $^{14}$C-glycerol into lipids is inhibited after exposure to cyanate for 1 hr at 37°C, even though virtually all unbound cyanate is subsequently washed out of the cells. Cyanate-treated cells do not increase their rate of lipid synthesis when the cell membrane is damaged by endocytotic agents such as the vinca alkaloids or puromycin. The rate of lipid synthesis in human reticulocytes which have been preincubated for 1 hr at 4°C with either 30 mM cyanate or normal saline, and subsequently exposed to $10^{-3}$ M puromycin or $1.5 \times 10^{-4}$ M vincristine after all of the unbound cyanate within the cell was washed out (see Methods), is shown in Fig. 4. The presence of puromycin inhibited incorporation of $^{14}$C-glycerol into lipids.

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**Fig. 2.** The effect of sodium cyanate on the incorporation of $^{14}$C-glycerol into human reticulocyte lipids. Reticulocyte-rich blood was incubated as described in Materials and Methods for 60 min at 37°C in the presence of concentrations of cyanate shown on the abscissa. The data are expressed as the percentage of radioactivity incorporated into whole lipid extracts of the cells, or isolated neutral lipids and phospholipids (separated by silicic acid chromatography), as compared to incorporation into cells incubated without cyanate. The blood was from a patient with autoimmune hemolytic anemia and had a reticulocytosis of 12%.
mycin or vincristine increases lipid synthesis to 192% and 187% of control values, respectively (shaded bar). Prior exposure of the cells to cyanate inhibits the response associated with membrane damage (hatched bar), although, when compared to the data shown in Fig. 2, the response is not completely abolished.

**Carbamylation of Membrane Proteins**

Since the irreversible effects of cyanate on the erythroid cell membrane are most likely due to carbamylation of membrane proteins, the relative rates of carbamylation of globin and membrane proteins under various conditions were investigated by comparing the proportion of the total protein-bound 14C-cyanate in the washed membranes to that of membrane-free hemolysate (Table 2). The absolute amount of cyanate bound irreversibly to membrane proteins increases with temperature, time of exposure, and concentration of cyanate, but...
Table 2. Carbamylation of Erythrocyte Membrane Proteins

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Per Cent of Total Cyanate cpm Attached to Membrane Protein at Cyanate Concentrations of</th>
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<tbody>
<tr>
<td></td>
<td>15 mM</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
</tr>
<tr>
<td>25</td>
<td>0.29</td>
</tr>
<tr>
<td>37</td>
<td>0.37</td>
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Cells were incubated at the indicated temperatures with three different concentrations of cyanate for 1 hr. After three washes in 310 mOsm phosphate buffer the membranes were separated from the hemolysate and washed three times again in 20 mOsm phosphate buffer. The counts per minute of 14C-cyanate in acid-precipitable protein of the membrane is expressed as the per cent of total acid-precipitable cpm in the cells.

under all conditions less than 1.5% of the total cyanate in the cell was bound to membrane protein. The rate of globin carbamylation, compared to that of membrane proteins, increased more rapidly with increasing concentrations of cyanate at a constant temperature, and with increasing temperature at a constant concentration of cyanate (Table 2). The proportion of cyanate bound to membrane proteins, indicating the rate of carbamylation in relation to that of globin, was decreased by incubating at lower temperatures. For example, the rate of carbamylation of membrane protein, in comparison with that of globin, after exposure to 15 mM cyanate was halved by decreasing the temperature of incubation from 37°C to 4°C. These data confirmed previous findings7 that by altering the temperature of exposure it was possible to emphasize the disproportion in rates of carbamylation of globin and membrane proteins.

Since in vivo survival of cells carbamylated with 15 mM cyanate at 4°C for 1 hr was not significantly different from normal (Table 1), in contrast to the decreased survival of cells exposed under harsher conditions, the findings suggested that limiting membrane carbamylation also limits membrane damage.

Carbamylation In Vitro

Experiments were designed to determine whether effective degrees of globin carbamylation could be obtained at levels of cyanate which only minimally damage erythrocyte membrane metabolic pathways. Figure 5A shows the degree of carbamylation of globin obtained with different concentrations of cyanate at 4°C for 60 min. The degree of carbamylation achieved with 15 mM cyanate, 0.5 moles CNO/mole of hemoglobin, is associated with an increased life span of sickle cells in oral and in vitro trials.2,5,8 These data indicate that a clinically effective level of carbamylation is obtainable at a concentration of cyanate that only minimally damages membrane metabolic functions. Carbamylation of globin is also temperature dependent (Fig. 5B). Increasing the temperature of carbamylation from 4°C to 22°C results in only 17% greater carbamylation; increasing the temperature to 37°C results in a more significant increase. The carbamylation reaction is rapid and irreversible. Exposing the cells to cyanate for periods longer than 30 min, at any temperature or concentration, does not substantially affect the ultimate degree of carbamylation.

The effect of commercially available anticoagulants on the degree of carbamylation achieved by incubating blood at 4°C with varying concentrations of
CARBAMYLATED ERYTHROCYTES

Fig. 5. (A) Carbamylation of hemoglobin as a function of cyanate concentration. Heparinized human whole blood was incubated with 7.5–30 mM sodium cyanate at 4°C for 60 min. (B) Carbamylation of hemoglobin as a function of temperature. Cells were incubated at the indicated temperatures with ACD anticoagulant in the presence of 15 mM cyanate for 60 min.

cyanate was also studied. Both heparin and ACD were equally effective in supporting the carbamylation reaction, but CPD was considerably less effective.

DISCUSSION

Extracorporeal carbamylation of blood of patients with sickle cell anemia offers a more direct and less toxic means of preventing sickling than does oral cyanate therapy. However, even under in vitro conditions carbamylation is not innocuous. Cyanate modifies both protein and glucose metabolism in erythroid cells. The present studies show that cyanate also affects the ability of the erythroid cell membrane to synthesize lipids. Since erythrocyte ATP levels are unaltered by cyanate, it is unlikely that the observations are due to inhibition of energy metabolism. At least part of the effect of cyanate on erythrocyte membrane metabolism is a permanent defect, presumably due to irreversible interaction of cyanate with the amino groups of membrane proteins. This type of action is most likely responsible for the effect of cyanate in decreasing glucose-6-phosphate dehydrogenase activity in erythroid cells. Both laboratory findings and clinical observations in human pathologic conditions and in experimental vitamin E-deficient rat systems have suggested that lipid synthesis is a membrane repair mechanism of physiologic significance. If this is the case, impairment of this system could be of particular importance in cells whose membranes may be already damaged by the sickling process. However, the relationship between the ability of the reticulocyte membrane to synthesize lipids and protection of the cell under stress has not yet been proved.

The definitive evidence that in vitro exposure to cyanate damages the erythroid cell is the decreased in vivo life span of the carbamylated cells. Although previous studies reported a normal life span of carbamylated red cells, these cells were exposed only to trace amounts of cyanate, rather than pharmacologic doses. The clinical evidence relating to the effect of cyanate on sickle erythrocyte life span has indicated an overall increased survival of carbamylated cells, but in no study have the treated cells been restored to a
fully normal life span.\textsuperscript{12,18,26} The present findings suggest that the advantageous effect of carbamylation in decreasing sickling may be counterbalanced by deleterious effects on the cell membrane which decrease cell life span.

Since it is likely that the irreversible effects of cyanate on membrane metabolism are due to carbamylation of membrane proteins, an effort was made to limit this particular reaction. Previous studies have indicated that the relative rates of carbamylation of membrane proteins and globin can be varied by changing the temperature of incubation.\textsuperscript{7} Our studies confirm this finding and indicate that the rate of membrane protein carbamylation, relative to that of globin, is decreased under two conditions: higher concentrations of cyanate and lower temperatures of exposure. Since the absolute amount of carbamylation of membrane protein with higher concentrations of cyanate is shown by our studies to damage the erythroid membrane, it was decided to limit membrane carbamylation by using low concentrations of cyanate at low temperature. By carbamylating with 15 mM cyanate at 4°C for less than 1 hr, the life span of cells was not significantly altered from normal, while globin carbamylation reached levels of 0.5 moles CNO/mole hemoglobin. Hematologic improvement of patients with sickle cell anemia, as evidenced by decreased reticulocyte count, increased erythrocyte life span, and increased level of circulating hemoglobin has been obtained with this level of globin carbamylation.\textsuperscript{2,5,18}

An important implication of these studies is that in vitro carbamylation can be easily adapted to the environment of the working blood bank. Commercial plastic bag plasmapheresis sets are available with either ACD or CPD as the anticoagulant, and separation of cells in the approved plasmapheresis procedure is done at 4°C.\textsuperscript{27} Since our studies show that adequate carbamylation of globin can be achieved at this temperature, and that maximum carbamylation is achieved after only 30 min of exposure, the standard plasmapheresis procedure can be used with minimal modification.\textsuperscript{27} The use of CPD as an anticoagulant is to be avoided, since through an effect not totally related to pH carbamylation of globin is inhibited by this anticoagulant mixture. Since anticoagulation with heparin offers no advantage over ACD, which is available in commercial plasmapheresis packs, the hazards of heparinizing the patient can be avoided.

Two clinical trials with in vitro carbamylation of sickle cell hemoglobin are currently in progress.\textsuperscript{5,6} The conditions of carbamylation in these trials differ widely, with the concentration of cyanate ranging from 24 to 35 mM, the conditions of exposure from 1 hr at 37°C to 24 hr at 4°C, and use of either heparin or ACD as an anticoagulant. Although levels of carbamylation of 1.5 mole CNO/mole hemoglobin were obtained in both studies, the effects of these conditions on the red cell were not studied. The present study does not indicate that carbamylation in vitro of erythrocytes is not an effective treatment for sickle cell anemia, but it does indicate that more adequate investigation of conditions to provide adequate carbamylation of globin while minimally damaging the red cell is justified. If other agents currently being considered as inhibitors of sickling eventually reach the stage of clinical trials, similar systematic studies to arrive at the optimal conditions for cell modification must be carried out.
REFERENCES


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