Reticulocyte Survival in Sickle Cell Anemia: Effect of Cyanate

By Blanche P. Alter, Yuet Wai Kan, and David G. Nathan

Cyanate prevents sickling in vitro and apparently prolongs the survival of $^{51}$Cr-tagged sickle erythrocytes in vivo. Cautious interpretation is required because the effects of cyanate on $^{51}$Cr binding to sickle and fetal hemoglobin-containing red cells are unknown, and comparison of the effect of cyanate on sickle red cell survival to control red cell survival must be performed sequentially. We have studied the survival of sickle reticulocytes utilizing radioactive amino acids that are incorporated into hemoglobin. Two informed adult patients with sickle cell disease were studied. In each study, two 50-mI samples of blood were incubated separately with $^{14}$C- and $^{3}$H-leucine for 2 hr, after which 50 mM cyanate was added to one aliquot for 1 hr. The cells were then washed and reinfused. Frequent venous samples were obtained, and the specific activities of $^{14}$C and $^{3}$H in the hemoglobin were followed. The $t_{1/2}$ of the carboxylated cells was tripled, but remained below normal. This method provides a generally useful measurement of the influence of drugs bound to red cells on reticulocyte lifespan. The labels are incorporated into the hemoglobin molecule of the reticulocyte, and simultaneous comparison of the survivals of the same cohort of drug-treated and control cells is achieved.

Cyanate inhibits sickling in vitro, both directly and by interference with oxygen release from hemoglobin, and prolongs the survival of $^{51}$Cr-labeled sickle erythrocytes in the circulation. Although prolongation of $^{51}$Cr survival curves suggests improvement of mean red cell survival, several theoretical and actual problems render precise interpretation difficult. These include the marked heterogeneity of circulating red cells in sickle cell anemia, the requirement for sequential $^{51}$Cr studies despite temporal variability of the hemolytic rate, and the potential influence of a drug such as cyanate on the in vivo elution of $^{51}$Cr.

A more precise method for evaluation of the effects of a drug on red cell survival in sickle cell anemia should meet the following criteria: (1) The drug should be incubated with an aliquot of erythrocytes in vitro to avoid administration of a potentially toxic agent directly to the patient. (2) Simultaneous survival curves of control and treated populations of cells should be

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obtained by the use of identical red cell markers that differ only with respect to their radioactive isotopes. (3) The label should be preferentially incorporated into reticulocytes to provide a "cohort" marker. (4) The label should not elute from the red cells, and the drug to be studied must not interfere with the binding of the label.

To achieve these requirements, we have incubated aliquots of reticulocytes with $^{14}$C- or $^3$H-leucine, treated one of these aliquots with cyanate, and compared the survival of the two populations directly. We have confirmed the improvement in sickle erythrocyte survival by 50 mM cyanate, although the reticulocyte lifespan is not restored to normal.

**MATERIALS AND METHODS**

Two adult patients with sickle cell disease were studied after informed consent was obtained. Their respective hemoglobins were 9 and 8 g/100 ml, reticulocyte counts were 17% and 21%, and hemoglobin S was 90% with no hemoglobin A present.

One hundred milliliters of heparinized blood was drawn and divided into two equal aliquots in Fenwal blood transfer bags (Fenwal Lab., Morton Grove, Chicago, Ill.). All additions to the blood were sterilized by passage through 0.22 μm Millipore filters (Millipore, Bedford, Mass.). For patient B.W., the aliquots were thrice washed at 4°C in a pH 7.5 buffer (0.05 M Tris-HCl, 0.005 M KCl, 0.1 M NaCl, 0.02 M NaHCO$_3$, 0.005 M MgCl$_2$). Each aliquot was then resuspended in 30 ml of the same buffer to which were added as final concentration 0.25 mM each of 19 amino acids minus leucine, and 8 $\times$ 10$^{-5}$ M Fe(NH$_4$)$_2$SO$_4$.6H$_2$O. For patient L.B., the initial washes were omitted, and the incubations were performed in plasma. In each case, glucose was added to a final concentration of 10 mM. To one aliquot from each patient was added 250 μCi of uniformly labeled $^{14}$C-leucine (New England Nuclear, Boston, Mass., specific activity 300 mCi/m mole), and 1 mCi of $^3$H-leucine (New England Nuclear, Boston, Mass., specific activity 37 Ci/m mole) was added to the other aliquot. Following oxygenation, the cells were incubated at 37°C for 2 hr to allow incorporation of the radioactive amino acids into the hemoglobin newly synthesized by the reticulocytes. Subsequent analysis revealed that approximately 25% of the radioactive amino acids were incorporated into hemoglobin.

At the end of 2 hr, sodium cyanate (Pfaltz and Bauer, Flushing, N.Y.) (0.5 M in isotonic saline) was added to one aliquot from each patient to a final concentration of 50 mM. For B.W., the cyanate was added to the cells labeled with $^3$H-leucine, while for L.B. it was added to the cells labeled with $^{14}$C-leucine. A 2 ml sample of B.W.'s blood was separately incubated with $^{14}$C-cyanate (New England Nuclear, Boston, Mass., specific activity 6 mCi/m mole), in order to measure the amount of carbamylation. After an additional hour of incubation to allow carbamylation to occur, all the cells were washed three times with sterile physiologic saline, resuspended to the original hematocrit, and reinjected into the respective donor. A 1 ml sample of the infused blood was retained in order to measure the amount of radioactivity given to the patient and to assess the recovery of labeled cells in the circulation. The recovery was calculated from the volume and radioactivity of reinjected cells, the estimated red cell volume of the recipient, and the radioactivity in a known volume of recipient red cells.

Ten milliliter samples of venous blood were collected in heparin 30 min after the infusion and at intervals thereafter. The cells were thrice washed in saline and lysed according to the methods of Lingrel and Borsook. Ten milligrams of hemoglobin in 0.4 ml hypotonic lysing fluid were pipetted into a scintillation vial to which were added 1.5 ml of a 1:1 mixture of isopropyl alcohol and Protosol (New England Nuclear, Boston, Mass.) and 1 ml of 30% H$_2$O$_2$. After bleaching for 30 min at room temperature, 10 ml of Aquasol (New England Nuclear, Boston, Mass.) was added, and the sample was counted for $^3$H and $^{14}$C in a Packard Model 3375 liquid scintillation counter (Packard Instrument, Downers Grove, Chicago, Ill.). Quenching was corrected with standard quench curves utilizing an automatic standardization technique.
The hemolysate labeled with $^{14}$C-cyanate was passed over a Sephadex G-25 column in order to determine the amount of cyanate bound to hemoglobin. The hemoglobin was isolated, and 1 mg in 0.5 ml was added to a counting vial, bleached with 0.5 ml of 30% $\text{H}_2\text{O}_2$, 1% ammonium bicarbonate and was counted in Aquasol as above. Approximately 3 moles of cyanate were bound per mole of hemoglobin tetramer.

Globin chains were precipitated from the hemolysate of patient B.W. on days 8, 20, and 71 of the study. One hundred milligrams of the chains were separated on CM-cellulose columns in phosphate buffer pH 6.7 of increasing ionic strength, and their radioactivities were determined. The relative survivals of globin chains derived from carbamylated and noncarbamylated cells were then assessed by integration of the areas under the radioactive alpha and beta-S peaks. For example, that total area under the $^{14}$C alpha and beta-S peaks observed on day 20 and day 71 was compared to the area observed on day 8, and a ratio of day 20 or day 71 counts to day 8 counts was calculated.

RESULTS

The recovery of labeled reticulocytes in both patients was virtually 100% 30 min after injection of the labeled cells. The ratio of $^3\text{H} : ^{14}\text{C}$ at 30 min was essentially that of the injected cells, indicating no early destruction of a population of cells damaged by cyanate. The survival of the cyanate-treated and untreated cells in both patients is shown in Fig. 1. Clearly, cyanate markedly increased the survival in both. The 50% survival time of untreated, newly formed cells was 14 days in B.W. and 10 days in L.B. In vitro incubation with cyanate prolonged these times to 50 and 28 days, respectively. In both patients, the survival curves were nonlinear on a semilogarithmic scale, indicating that the curves represent composites of survivals of red cells that are removed at variable rates. Curves representing the survival of carbamylated and noncarbamylated cells initially rose slightly above the
30-min value in patient B.W. and fell at a very slow rate for a short time in L.B. This suggests that some of the labeled cells might have been sequestered temporarily and then slowly returned to the circulation.

Separation of globin chains on CM-cellulose columns demonstrated that incubation with cyanate led to the formation of additional $^3$H-labeled globin peaks, which eluted from the column earlier than the normal beta-S and alpha chains (Fig. 2). This is due to a change in the charge of the carbamylated chains. For this reason, the integrated area of the alpha and beta-S peaks was measured on the basis of four peaks (two each for alpha and beta-S chains) for the $^3$H-globin of the carbamylated cells, in contrast to two peaks for the $^{14}$C-globin from the noncarbamylated cells. The ratios observed at 20 and 71 days for $^{14}$C from globin chains from noncarbamylated cells were 0.50 and 0.12, respectively, whereas the same cyanate-treated $^3$H-labeled ratios were 0.72 and 0.29, respectively.

**Fig. 2.** CM-cellulose chromatograms of globin chains, patient B.W. Equal amounts of protein were applied to each column. Bottom panel shows absorbance at 280 nm, demonstrating location of beta-S and alpha chains. (A) day 8; (B) day 20; (C) day 71.
DISCUSSION

Assessment of the influence of drugs on the survival of sickle red cells poses a difficult technical problem. Of prime consideration is the requirement for in vitro labeling and drug exposure, which avoids the unacceptable hazard of experimental drug administration to human recipients. The sequential in vivo administration of labels such as $^{59}$Fe or $^{14}$C-glycine is therefore excluded.

Standard in vitro methods involving labeling of cells of all ages (Ashby, diisofluorophosphate (DFP)-$^{32}$P or -$^{3}$H, and Na$^{51}$ CrO$_4$) have several significant shortcomings. The Ashby method involves an unacceptable risk of transmission of hepatitis. The availability of $^{3}$H- and $^{32}$P-labeled DFP permits simultaneous survival studies of drug-treated and control cells, but the rapid in vivo elution of DPF from in vitro labeled cells limits interpretation of data in patients whose red cell life span is short. Na$^{51}$CrO$_4$ is convenient and widely used, but its use for the assessment of drug effect demands sequential studies. The inherent variability of $^{51}$Cr survival curves in sickle cell anemia and the possible effect of the drug on in vivo $^{51}$Cr elution reduce the validity of sequential observations. In addition, $^{51}$Cr labels the heterogeneous population of erythrocytes in sickle cell disease. These include, in addition to reticulocytes, irreversibly sickled cells and a variably sized cohort of fetal hemoglobin-enriched cells. Since the survival of the latter two populations would be largely unaffected by a drug designed to inhibit sickling, the contribution of these cells to the survival curve might be expected to obscure the effects of the drug on the lifespan of newly formed cells.

For these reasons, we have adopted an in vitro, cohort labeling system that permits simultaneous measurement of the survival of two populations of reticulocytes, one of which is subsequently exposed to cyanate. The choice of the labeling agents was influenced by the availability of $^{3}$H- and $^{14}$C-labeled amino acids, which, unlike $^{59}$Fe and $^{55}$Fe, reappear only slightly in newly formed cells after the completion of the life span of initially labeled cells. The labeled amino acids are of sufficient specific activity to permit adequate assessment of the radioactivity in 10 mg of hemoglobin during the simultaneous drug-treated and control survival study. The use of $^{14}$C-glycine for measurement of reticulocyte lifespan in pyruvate kinase deficiency has been previously described.

After exposure of labeled sickle reticulocytes to 50 mM cyanate for 1 hr, the lifespan of the cells was significantly prolonged. This was confirmed by analysis of the separated globin chains from larger aliquots of cells removed 8, 20, and 71 days after labeled cell administration. To be sure, the cyanate exposure did not produce a normal survival, with the lifespan limited by red cell senescence. Had the drug done so, the radioactivity of the carbamylated cells would have persisted at a fairly constant level for approximately 80 days and would then have declined in a sigmoidal fashion. Instead, the shapes of the survival curves of the carbamylated and noncarbamylated cells, although different, revealed persistent increased random destruction, albeit at different rates.
Noncarbamylated reticulocytes were removed at variable rates, a pattern of survival that is also observed when the total population of cells is labeled. We suggest that the most rapid rate of destruction reflects the population of cells that becomes irreversibly sickled and is promptly destroyed. The remainder (perhaps those with a higher concentration of fetal hemoglobin) are destroyed more slowly. Reutilization of label might also contribute to the deviation of the survival curve from the initial rate.

Carbamylation has as its most striking effect improvement of the survival of the most rapidly destroyed population, suggesting that the development of irreversibly sickled cells may be inhibited by this agent. Thus, the over-all of the survival curve was increased. The drug did not improve the survival of the more slowly destroyed population. In fact, the data (albeit scanty) suggest the opposite effect. This may be due to inhibition of red cell metabolism by the degree of carbamylation employed here, as suggested by the studies of Diederich et al., deFuria et al., Lowman and Diederich, and Glader and Conrad.

These studies of carbamylated reticulocyte survival in sickle cell disease confirm the results of Gillette et al. and provide a technique for further study of drug effects on sickle cell lifespan. However, the prolongation of survival observed here and by Gillette must be interpreted with caution. Fifty millimolar cyanate is an unacceptably toxic concentration of this drug, as evidenced by ongoing studies of protein synthesis in sickle and rabbit reticulocytes and sickle bone marrow. Five-millimolar concentrations of the drug significantly inhibit protein synthesis in vitro, whereas such concentrations do not inhibit in vitro sickling. For this reason, it will be necessary to establish the effect of cyanate on sickle cell lifespan at concentrations of the drug at which sickling is inhibited and at which toxic manifestations are significantly reduced.

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