Time-Dependent Loss of Adenosine 5'-Monophosphate Deaminase Activity May Explain Elevated Adenosine 5'-Triphosphate Levels in Senescent Erythrocytes

By George L. Dale and Shannon L. Norenberg

Senescent erythrocytes from rabbits were previously shown to have elevated levels of adenine nucleotides. The present study documents that aged red blood cells have a normal synthetic capacity for adenine nucleotides, as indicated by normal levels of adenosine kinase. However, senescent erythrocytes do have decreased levels of adenosine 5'-monophosphate deaminase, the critical enzyme involved in degrading adenine nucleotides. These circumstances of a normal synthetic capacity in the presence of decreased catabolic ability were observed previously in a human genetic deficiency of adenosine 5'-monophosphate deaminase; the red blood cells in these patients accumulate adenosine 5'-triphosphate as do senescent erythrocytes in rabbits.6

THE MECHANISMS that govern erythrocyte senescence are unknown despite several decades of active investigation.7 The explanation for this paucity of data is the difficulty of reliably isolating aged red blood cells (RBCs).8,9 Several recent techniques allow isolation of senescent erythrocytes and have therefore greatly facilitated investigations in this field.10,11 In this study, one of these methods9 was used to examine the state of adenine nucleotide metabolism during erythrocyte senescence.

The isolation method used for aged RBCs involves the in vitro biotinylation of rabbit erythrocytes. These derivatized cells are reinfused into the original donor animal and subsequently isolated after in vivo aging.12 This technique was recently used to examine a variety of parameters in aged erythrocytes,13,14 including the level of adenine nucleotides in senescent cells. These previous studies with senescent erythrocytes isolated by the biotinylation method demonstrated the rather unexpected finding that adenosine 5'-triphosphate (ATP) levels are elevated approximately 75% above normal as the rabbit RBC reaches the end of its 60-day lifespan.15 This result contradicts earlier reports that senescent erythrocytes which lack AMP deaminase completely do not accumulate even higher amounts of ATP.16 The critical role of AMP deaminase in controlling adenine nucleotide levels is exemplified by an inborn error of metabolism where the enzyme is absent from erythrocytes; these RBCs have elevated levels of ATP.16 Therefore, in the absence of this key catabolic activity, erythrocytes continue to synthesize AMP and accumulate ATP. Another catabolic pathway for degradation of AMP in RBCs uses purine 5'-nucleotidase. The role of this activity in the erythrocyte is controversial17,18, however, the presence of this additional catabolic pathway may explain why erythrocytes which lack AMP deaminase completely do not accumulate even higher amounts of ATP.

The present study examined the status of adenine nucleotide metabolism in senescent erythrocytes from rabbits. The aged RBCs had decreased activity of AMP deaminase, which may explain the elevated ATP levels observed in these cells.

MATERIALS AND METHODS

N-Acyxyoxysuccinimido-biotin was obtained from Pierce Chemical, Rockford, IL. Avidin was from Calbiochem, La Jolla, CA. Amersham, Arlington Heights, IL, supplied the 14C-AMP. All other biochemicals were obtained from Sigma Chemical, St Louis, MO. Polyethylene Petri dishes (D1905) were from Scientific Products, McGaw Park, IL.

Isolation of aged rabbit erythrocytes. New Zealand White rabbits (2.0 to 2.5 kg) were injected subcutaneously on day 13 with 15 mg phenyldrazine hydrochloride dissolved in 150 mmol/L Tris, pH 8.7; the injections were repeated on days 12 and 11. On day 0, approximately 50 mL blood was withdrawn from the rabbit into heparin and biotinylated as previously described16 except that the cells were not labeled with 14C-cyanate. The biotinylated erythrocytes were reinfused into the original donor rabbit.

At various times after reinfusion, blood was drawn into heparin, the leukocytes were removed,19 and the RBCs were washed three times in 150 mmol/L NaCl and 10 mmol/L Na phosphate, pH 7.5 (phosphate-buffered saline [PBS]). The erythrocytes were resuspended to a 33% hematocrit in PBS containing 1 mg/mL bovine serum albumin and 5 mmol/L glucose. Three milliliters of this mixture was applied to each avidin plate (described below); the mixture was complete, the plates were washed gently with 150 mmol/L glucose. Three milliliters of this mixture was applied to each avidin plate (described below); the mixture was complete, the plates were washed gently with 150 mmol/L NaCl. To remove the bound, biotinylated erythrocytes from the plate, 5 mL of PBS containing 350 U of collagenase, 1 mg/mL bovine serum albumin and 0.1 mmol/mL biotin4 was then added, and the plate was rocked at room temperature for 10 minutes. Some

From the Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, CA. Submitted April 4, 1989; accepted June 6, 1989.

Support for this work was provided by Grant No. AG 08545 from the National Institutes of Health, Bethesda, MD. This is publication number 5552-MEM from the Research Institute of Scripps Clinic.

Address reprint requests to George L. Dale, PhD, Research Institute of Scripps Clinic, BCR-3, 10666 N Torrey Pines Rd, La Jolla, CA 92037.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.
Twelve milliliters of starting buffer was then used to wash the RBCs equilibrated with 0.72 mmol/L 2-mercaptoethanol, pH 7.

The validity of the isolation procedure is routinely verified with a radioisotopic assay. One hundred microliters of a 1:20 hemolysate was added to 100 μL reaction mixture (40 mmol/L AMP, 150 mmol/L KCl, 0.5 mmol/L EDTA, 3 mmol/L 2-mercaptoethanol, and 10 mmol/L phosphate, pH 7.0 with 400,000 cpm of 14C-AMP/mL) and incubated at 37°C for 15 minutes. The reaction was stopped with 2 mL 20 mmol/L glycine-HCl, pH 2.2, and the entire mixture was applied to a 1.0-mL column of Dowex-1 equilibrated with the 20 mmol/L glycine-HCl, pH 2.2. Twelve milliliters of starting buffer was then used to wash the unadsorbed AMP substrate off the column. The 14C-inosine 5'-monophosphate product was then eluted from the column with 3 mL 0.5 mol/L HCl, and the radioactivity was quantitated by scintillation counting.

Adenosine kinase was assayed by the method of Kyd and Bagnara, and hemoglobin was quantitated by absorbance at 410 nm.

RESULTS

Rabbits were treated with phenylhydrazine to produce a young cohort of erythrocytes; these cells were then derivatized with biotin and reinfused into the original donor animals. At various times after the reinfusion, the animal was again bled and the biotinylated cells were isolated by their affinity for an avidin support. This technique allows an unambiguous isolation of various aged erythrocytes and thereby facilitates examination of the factors that may be involved in RBC senescence.

Aged erythrocytes were isolated and examined for the levels of two enzymes involved with adenine nucleotide metabolism. The activity of adenosine kinase measured in these senescent erythrocyte samples is shown in Fig 1; the data indicate that this synthetic enzyme activity is not dramatically affected by the aging of RBCs.

Figure 2 shows the AMP deaminase activity for aging erythrocytes from rabbits. The AMP deaminase activity quickly decreases during the first 40 days of the erythrocyte lifespan and then stabilizes for the remaining 20 days. The final level of AMP deaminase was approximately 30% of control value. The control samples assayed were unfractionated cells which contained approximately 4% to 5% reticulocytes, and reticulocytes have quite high levels of AMP deaminase. Therefore, the true level of AMP deaminase in a control population of mature RBCs will be slightly lower than the 9.67 IU/g hemoglobin shown in Fig 2.
AMP DEAMINASE IN AGED RED CELLS

DISCUSSION

Previous experiments showed that aged erythrocytes from rabbits have abnormally high levels of ATP. The purpose of this study was to determine if the regulatory mechanisms that control adenine nucleotide pool size were abnormal in senescent RBCs.

Our data document that aged erythrocytes from rabbits have normal levels of adenosine kinase and decreased levels of AMP deaminase. An impairment of AMP deaminase would result in decreased adenine nucleotide catabolism and, if synthesis continued unabated, a net increase in adenine nucleotides might result. Indeed, an excellent correlation exists between the timing for loss of AMP deaminase activity and the increase in ATP levels. As shown in Fig 2, the AMP deaminase activity reaches a minimum at 40 days, which corresponds with the first observable increase in ATP levels. Specifically, the earlier report showed that RBC ATP levels at 42, 50, and 60 days after biotinylation were 134%, 152%, and 176%, respectively, of control values. This steady increase in ATP concentration presumably reflects the continued synthesis of adenine nucleotides by adenosine kinase in the absence of a normal degradative process. A similar situation occurred with a genetically determined absence of AMP deaminase in which RBCs were also shown to have increased quantities of ATP.

Paglia et al demonstrated that aged erythrocytes obtained from children with transient erythroblastopenia also have decreased levels of AMP deaminase and increased concentrations of adenine nucleotides. These findings suggest that the observed loss of AMP deaminase with erythrocyte age may be a general phenomenon. However, the increased ATP levels or decreased AMP deaminase activity observed for aged RBCs are probably not detrimental to the cell’s function and are not prime determinants of the cellular lifespan. This conclusion is based on several mutations in humans which result in increased erythrocyte ATP levels with no untoward effect on the cell’s viability.

The time-dependent loss of erythrocyte AMP deaminase activity is easily documented in rabbits in which many time points can be obtained during the 60-day lifespan of these cells. However, the pattern of decline in AMP deaminase activity is not what would be generally expected for a degradative process in the RBC. Most investigators propose that a progressive loss of enzyme activity throughout the entire cellular lifespan or a rapid loss of enzyme during reticulocyte maturation might be anticipated. The data we present suggest that the decrease in activity is complete within the first 40 days of the cell’s life, with little if any further decrease during the last 20 days of circulation. This pattern might be explained if multiple isozymes of AMP deaminase were present; however, Ogasawara et al reported that human erythrocytes, at least, contain a single isoenzyme of AMP deaminase. This finding is supported by genetic studies in which complete loss of the enzyme occurs in families inheriting an autosomal recessive gene. With this background, it is possible that the AMP deaminase in rabbits is being destroyed by a proteolytic system which is itself dying off with time in the circulating RBC and, as the protease is lost, the remaining AMP deaminase is spared degradation. Alternatively, the AMP deaminase may be covalently modified during the RBC lifespan, resulting in an enzyme with decreased activity. Examination of the residual AMP deaminase present in the aged erythrocyte of rabbits may allow some conclusions as to the mechanisms that control the loss of this enzyme with time.

REFERENCES

8. Kirkpatrick FH, Muhs AG, Kostuk RK, Gabel GW: Dense (aged) circulating red cells contain normal concentrations of adenine triphosphate. Blood 54:946, 1979
20. Dale GL, Norenberg SL: Density fractionation of rabbit erythrocytes results in only a slight enrichment for aged cells (submitted)


Time-dependent loss of adenosine 5'-monophosphate deaminase activity may explain elevated adenosine 5'-triphosphate levels in senescent erythrocytes

GL Dale and SL Norenberg