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Use of Alveolar Carbon Monoxide to Measure the Effect of Ribavirin on Red Blood Cell Survival

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A major side effect of ribavirin (RBV) treatment is anemia. While this anemia is thought to result from increased RBC turnover, RBC survival has not been determined in subjects receiving RBV due to the complexity of the techniques commonly used to quantitate RBC life span. We recently described a simple, rapid, non-invasive technique that utilizes measurements of alveolar carbon monoxide (CO) concentration to determine RBC survival. In the present report, this method was employed to assess RBC survival in patients receiving RBV for hepatitis C. Each of the 31 measurements of RBC survival in 12 subjects with RBV-associated anemia was below the lower limit of normal (77 days), and the average survival (46 ± 14 days) in these subjects was only about 38% of that of healthy controls (122 ± 23 days). Five hepatitis C patients not undergoing RBV treatment had normal RBC survivals (112 ± 17 days). While the mean reticulocyte percentage was significantly elevated in subjects treated with RBV, 59% of these measurements fell within the limits of normal. We conclude that RBV-associated anemia consistently is associated with reduced RBC survival as determined from breath CO measurements and that this reduced survival frequently is not associated with an elevated reticulocyte count. Am. J. Hematol. 76:107–113, 2004. Published 2004 Wiley-Liss, Inc.†

Key words: red cell life span; carbon monoxide; reticulocytes; ribavirin

INTRODUCTION

Combined therapy with interferon α (INF) and ribavirin (RBV) has become the standard form of treatment for patients with chronic hepatitis C. Ribavirin is a water-soluble synthetic analogue of guanosine that exerts its antiviral activity by inhibiting intracellular phosphorylation reactions [1]. While this activity is not deleterious to most human cells, erythrocytes (RBCs) are adversely affected [2–4], and the vast majority of subjects receiving RBV become anemic [5–7].

It is widely assumed that RBV-induced anemia primarily results from increased RBC turnover [6]. Ribavirin is concentrated in circulating RBCs [8], causing a relative adenosine triphosphate deficiency and increased susceptibility to oxidative damage [9]. These events are thought to cause membrane changes that result in premature removal of cells from the circulation [9].

Evidence that RBV-induced anemia results from increased RBC turnover is limited to the observation that the reticulocyte percentage tends to increase during RBV therapy [6]. The absence of more direct, quantitative evidence of increased RBC turnover appears to be attributable to the lack of an easy means of assessing RBC lifespan in the clinical situation. We recently described a simple, rapid test that utilizes breath carbon monoxide (CO) concentration to quantitate RBC survival [10,11]. In the present study, this technique was employed to quantitate the influence of RBV therapy on RBC survival.

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PATIENTS AND METHODS

Subjects

Eighteen non-smoking male subjects (age 42–65 years) with hepatitis C were studied. Thirty-four measurements of RBC survival were obtained in 13 subjects undergoing treatment for hepatitis C with 3 million units of interferon α-2b SQ three times a week and 600–1200 mg RBV PO daily (Schering Plough, Kenilworth, NJ). The 13 subjects had been receiving RBV therapy for 4–34 weeks at the time of study. Survival measurements also were obtained in five hepatitis C subjects who were not receiving RBV. The study was approved by the Institutional Review Board of the Minneapolis VAMC and was carried out according to the Declaration of Helsinki. Informed consent was obtained from all subjects.

Alveolar and Atmospheric Gas Sampling

The rationale and the mechanics of the technique employed to measure alveolar CO have been described in detail in a previous publication [10]. In brief, immediately upon awakening in the morning, subjects closed their nares with a nose pinch, inhaled a normal-size breath, placed the mouthpiece of a breath collection apparatus (AlveoSampler, Quintron Instruments, Milwaukee, WI) in their mouth, and sealed their lips around the mouthpiece. After a timed, 20-sec period of breath-holding, the subjects exhaled into the collection system, which automatically discards the first 500 mL (dead space) and directs subsequent alveolar air into a self-sealing foil bag. Immediately upon collection of a second, duplicate breath sample, the subjects aspirated an atmospheric sample from the bedroom into a 20-mL syringe, which was sealed with a stopcock. Samples were delivered to the laboratory for analysis, either directly or via the mail. Preliminary studies showed that the concentration of CO in the foil bag and syringe decreased by $< 5\%$ during the up to 2-day period required for mail delivery.

Gas Analysis

To ensure that alveolar samples were properly collected, breath samples were analyzed for CO_2 concentration via an infrared analyzer (CAPSTAR-100, CWE, Inc., Ardmore, PA). The rare sample that contained less than 5% CO_2 was discarded. The concentration of CO in alveolar and atmospheric samples was determined by gas chromatography using an instrument equipped with a 400-μL gas sampling valve, a column (6 ft × 1/8 inch) packed with 5-Å molecular sieve, and a reduction detector (Trace Analytical RGD2, Menlo Park, CA). The oven temperature was 110°C, and nitrogen was used as the carrier gas (40 mL/min). The CO concentration of the unknowns was determined via reference to peak areas of standards of known concentrations. Means of the results of the duplicate CO measurements of alveolar air were used in subsequent calculations.

Hematologic Measurements

Hemoglobin values and reticulocyte percentages were determined via a Coulter GEN-S apparatus. Blood was analyzed for reticulocyte percentage on 29 of the 34 occasions that CO measurements were obtained.

Calculations

Survival of RBCs based on CO measurements was calculated from [11]:

$$\text{RBC life span} = \frac{[\text{Hb}](22,400)(\text{blood volume})(4)}{(0.7)(\text{Endogenous } P_{\text{CO}})(64,400)(1,440)(\text{alveolar ventilation})}, \quad (1)$$

where 22,400 is the mL of CO/mol; 4 is the number of moles of heme per mole of Hb; 0.7 is the fraction of V_{CO} derived from Hb turnover; endogenous P_{CO} is alveolar P_{CO} minus atmospheric P_{CO} in ppm, 64,400 is the molecular weight of Hb; and 1,440 is min/day. Because blood volume and resting alveolar ventilation vary with body weight and in subjects with normal pulmonary function, both have roughly similar magnitudes when blood volume is expressed as mL and alveolar ventilation as mL/min, these two values cancel out in eq. (1). When [Hb] is in units of g/mL and endogenous P_{CO} is in ppm, eq. (1) reduces to the simple expression:

$$\text{RBC life span (days)} = \frac{[\text{Hb}]\ (1{,}380)}{\text{Endogenous } P_{\text{CO}}}. \quad (2)$$

Survival of RBCs based on the reticulocyte count was calculated from:

$$\text{RBC survival (days)} = \text{reticulocyte maturation time (days)/reticulocyte fraction.} \quad (3)$$

Reticulocyte maturation time in the circulation of healthy subjects was calculated from the normal mean value for RBC survival (120 days) and the mean reticulocyte fraction observed in healthy subjects (0.0108). Thus

$$\text{Reticulocyte maturation time} = (120 \text{ days}) \times (0.0108) = 1.30 \text{ days}. \quad (4)$$

Assuming a normal reticulocyte maturation time, the RBC survival of an individual can then be calculated from his/her reticulocyte fraction:

RBC survival (days) = 1.30/reticulocyte fraction. (5)

All results are expressed as mean ± 1 SD.

RESULTS

Figure 1 plots the RBC life span (calculated from CO measurements) against the hemoglobin concentration for the 34 observations made in 13 hepatitis C subjects being treated with INF and RBV as well as for the five observations obtained in five hepatitis C subjects who were not receiving RBV. The normal range of RBC survival previously determined in 40 healthy controls using the breath CO technique [10] is also indicated. All hemoglobin measurements in 12 of the 13 RBV treated subjects (non-starred triangles) were < 13.5 g/dL, averaging 11.9 ± 1.1 g/dL. Each of the 31 measurements of RBC survival obtained in these 12 subjects was at or below the lower limit of normal. The mean RBC survival of this group was 46 ± 14 days, 38% of the normal survival, which averages 122 ± 23 days [10]. One of the 13 RBV-treated subjects (indicated by triangles with a star in Fig. 1) had hemoglobin concentrations >14 g/dL on three separate occasions, and the three measurements of RBC survival in this subject fell within the normal range. The five hepatitis C subjects not receiving RBV each had normal RBC survivals that averaged 112 ± 16 days (see Fig. 1). The correlation coefficient of hemoglobin concentration versus RBC survival in RBV-treated subjects was 0.41 ($P < 0.02$).

Figure 2 shows a plot of reticulocyte percentage versus hemoglobin concentration measured on 29 occasions in 11 subjects being treated with RBV. The normal limits for reticulocyte percentage (0.6–2.1%) are indicated. The reticulocyte counts of these subjects, all of whom had hemoglobin concentrations <13.5 g/dL and reduced RBC life spans (measured via CO), averaged 1.88 ± 0.60%. While this value is significantly greater ($P < 0.0001$) than the normal mean of 1.08% for healthy subjects, reticulocyte counts fell within the normal range for 17 of the 29 observations. The correlation coefficient between reticulocyte percentage and hemoglobin concentration was 0.12 ($P > 0.10$).

Figure 3 plots RBC survival calculated from CO versus RBC survival calculated from the reticulocyte count. In 26 of 29 measurements, RBC survival determined from reticulocyte count was greater than the value calculated from CO measurements. Life span calculated from reticulocyte fraction averaged 77 ± 28 days versus 46 ± 14 days for the CO-based measurement ($P < 0.0001$).

DISCUSSION

Erythrocyte survival seldom is quantitated in subjects with presumed hemolytic anemia due to the complexity of the methodology required for measurement

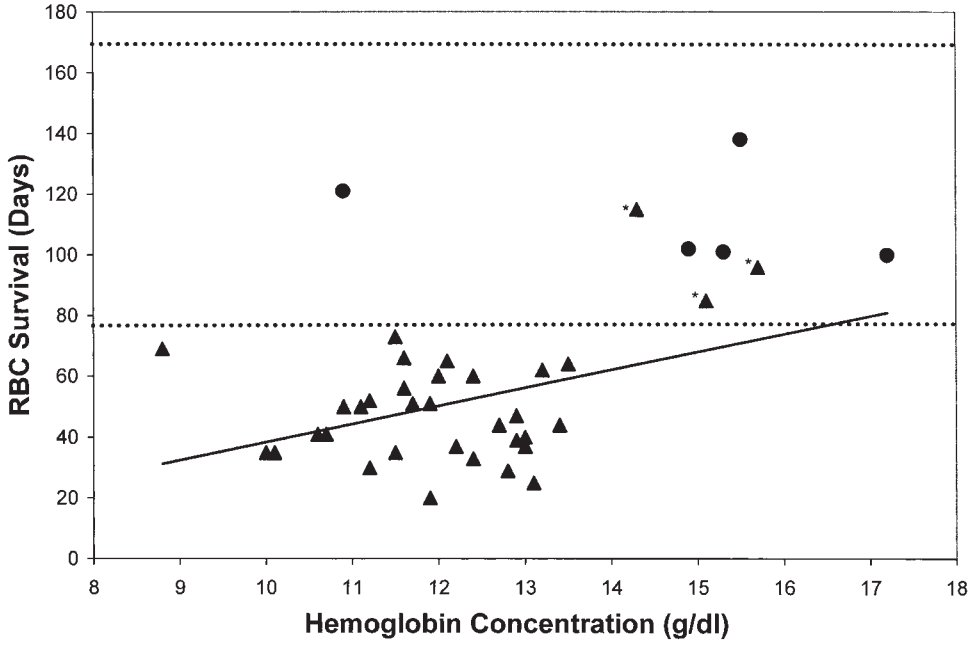

Fig. 1. Relationship of CO-calculated RBC survival to hemoglobin concentration in hepatitis C subjects. (▲) Observations in RBV-treated hepatitis C subjects. (*▲) Three observations in the one hepatitis C subject whose RBC survival was unaffected by RBV therapy. (●) Hepatitis C patients not receiving RBV. (---) Normal limits (mean ± 2 SD) for RBC survival in healthy individuals. The correlation coefficient between RBV survival and hemoglobin concentration was 0.41 ($P < 0.02$).

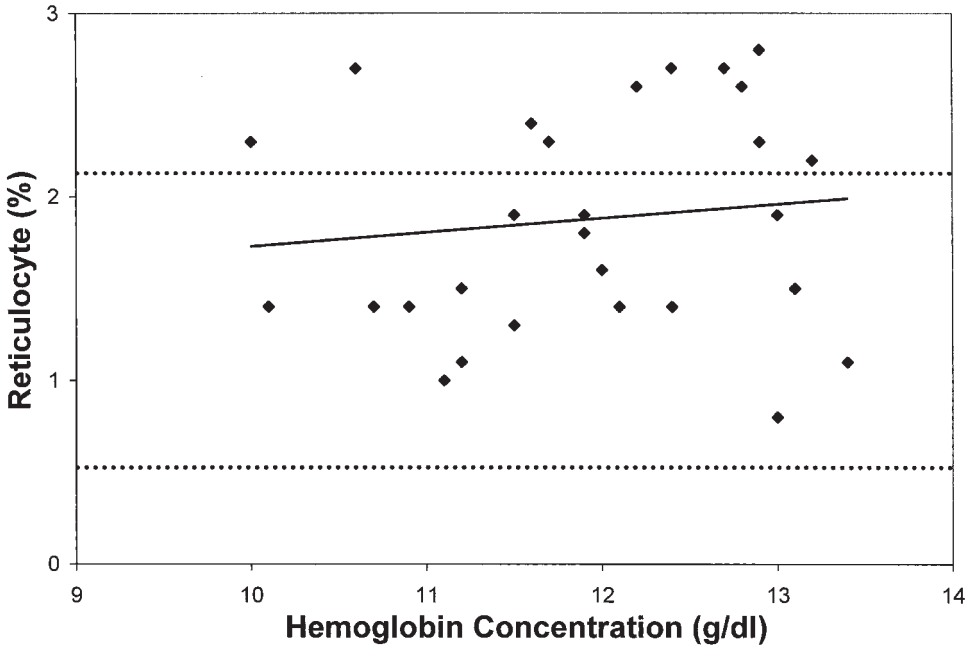

Fig. 2. Relationship of reticulocyte % to hemoglobin concentration in RBV-treated hepatitis C subjects. The correlation coefficient (r = 0.12) was not statistically significant. The dashed lines indicate the normal limits (mean ± 2 SD) for reticulocyte percentage in healthy individuals.

of RBC survival. The "gold standard" technique for quantitating RBC survival requires the administration of a label that is incorporated into RBCs at the time of their formation in the bone marrow. The disappearance of the tagged cells from the circulation is then monitored, a process that requires a follow-up period of more than 120 days for subjects with normal survival [12,13]. The only methodology employed with any frequency in the clinical situation involves labeling of circulating RBCs (most commonly with ^{51}Cr) and then following the disappearance of the labeled cells from the circulation [14,15]. In addition to inaccuracies resulting from elution of the label, this method requires repeated venesections over a multi-week period to obtain a single survival measurement. The complexity, radioactive exposure, and time commitment of this technique markedly reduces its clinical utility. As a result, available evidence that the anemia of RBV-treated subjects results from rapid turnover of RBCs consists solely of the demonstration of increased reticulocyte counts in some subjects [1–3,6,7].

In the present study, measurements of breath CO concentration were used to measure RBC survival. Nearly all CO generated in the body is derived from the α-methene carbon of heme, which is stoichiometrically released as CO when the α-methene bond is cleaved in the process of converting heme to bilirubin [16]. The vast majority of heme turnover results from RBC destruction; thus, the rate of CO production can be used as a quantitative indicator of RBC turnover rate. Measurements of expired CO provide a convenient means of assessing CO production since this gas is cleared entirely via the lungs [17]. However, breath CO originating from heme metabolism (endogenous CO) must be distinguished from CO of environmental origin (exogenous CO). Initial reports using CO to assess RBC turnover required that subjects rebreathe into a closed system for several hours to distinguish exogenous from endogenous CO [18–20]. In the present study, we utilized a simplified technique in which the difference between the CO concentration of alveolar air and atmosphere was used to quantitate endogenous CO excretion rate (and RBC survival) [10]. Evidence of the validity of this technique includes its ability to demonstrate normal (approximately 120-day) RBC life spans in healthy subjects and reduced RBC life spans in subjects with hemolysis as documented by ^{51}Cr-labeling or fecal bile pigment measurements [11]. It should be emphasized that this technique is not applicable to smokers or subjects with impaired pulmonary function.

Multiple measurements obtained in 13 subjects undergoing RBV therapy showed that 12 of these subjects consistently had a reduced RBC life span that averaged 46 days, roughly 40% of the 122-day life span observed in healthy subjects ($P < 0.001$) [10]. Each of these subjects also had a mildly reduced hemoglobin concentration (<13.5 g/dL), with a mean value of 11.9 ± 1.09 g/dL. Assuming a normal hemoglobin concentration of about 15 g/dL for males of this age group, the mean hemoglobin concentration

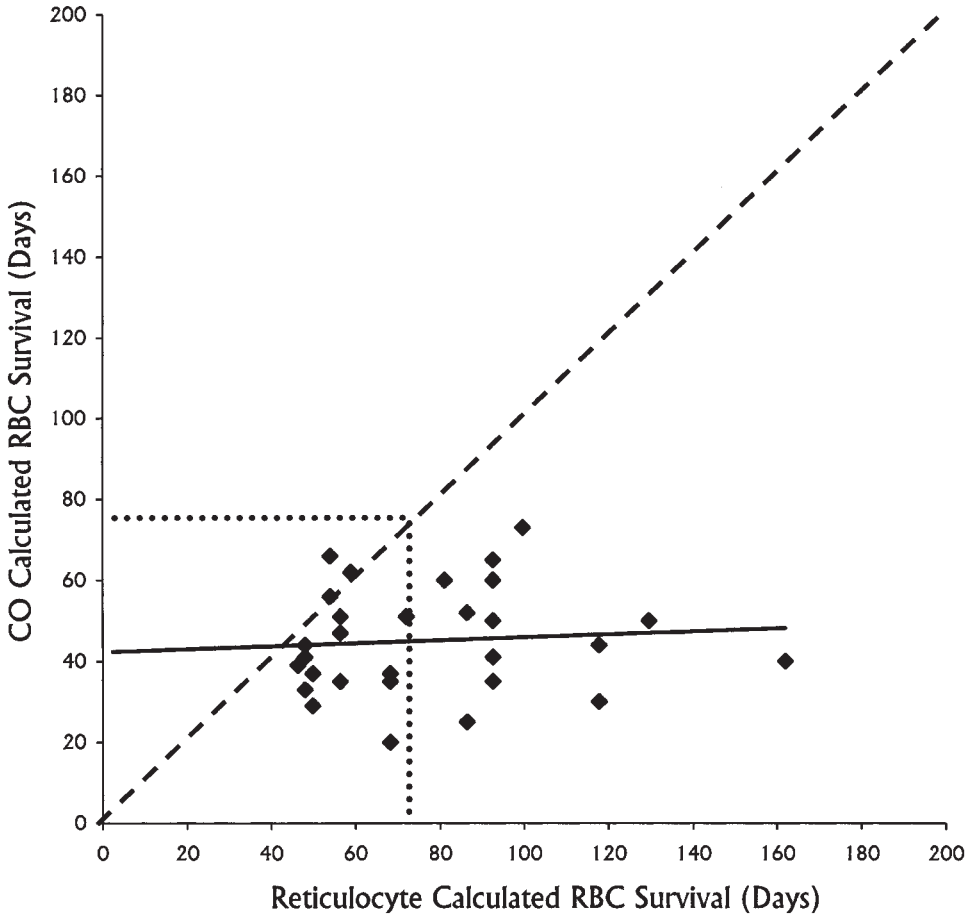

Fig. 3. Comparison of CO-based versus reticulocyte-based RBC survival measurements (see text for calculations). The solid line (—) represents the best fit to the data, while the dashed line (---) is the line of identity. The dotted rectangle indicates the lower limits of normal for RBC survival (determined from CO measurements). The correlation coefficient between the two measurements ($r = 0.08$) was not statistically significant. Note that in 26 of 29 observations, RBC survival was greater when calculated from the reticulocyte count versus breath CO and that 16 out of 29 survival measurements calculated from the reticulocyte count fell within the normal limits.

of these 12 RBV-treated subjects was reduced by about 20% as compared to the 60% decrease in RBC survival. Such a discrepancy is not surprising because the bone marrow would be expected to respond to increased RBC turnover with an increased rate of RBC output. The failure of RBC production to increase to the extent required to maintain a normal hemoglobin concentration may reflect a toxic effect of RBV on erythropoiesis, or a signal inadequate to stimulate the requisite RBC output, or bone marrow inhibition secondary to the concomitant interferon α therapy.

One of the 13 RBV-treated subjects presented an interesting anomaly in that his hemoglobin was consistently above 14 g/dL, and each of his three RBC lifespan measurements were in the normal range. The initial suspicion was that this subject might not be receiving RBV (which is self-administered orally two times a day). However, the patient adamantly maintained that he was using the drug as directed. The anomalous resistance of this subject's erythrocytes to the usual deleterious effect of RBV has been observed in previous reports that found that the occasional patient receiving RBV does not experience a fall in hemoglobin [1–3]. The physiology underlying this resistance remains speculative.

In contrast to the consistent reduction in RBC survival indicated by CO measurements in RBV-treated subjects, reticulocyte percentages remained within normal limits in 17 of 29 observations in these subjects. Thus, while physicians commonly rely on measurements of the reticulocyte count to assess RBC survival, this test appeared to be relatively insensitive at detecting the low-grade hemolysis of RBV-treated subjects.

In the steady state, where RBC production equals RBC destruction, the fraction of reticulocytes in the erythrocyte population relates to RBC survival via the expression:

$$\text{RBC survival (in days)} = \frac{\text{reticulocyte maturation time (days)}}{\text{reticulocyte fraction}}$$

Thus, the reticulocyte fraction is inversely proportional to RBC survival and should be an accurate indicator of survival providing that the reticulocyte fraction can be accurately measured and the reticulocyte maturation time is known. Until recently, the reticulocyte fraction was determined by an observer who hand-counted reticulocytes present in microscopic fields involving, at most, 1,000 erythrocytes. The limited number of reticulocytes counted and human error limited the accuracy of this technique. The presently employed automated techniques scan many thousands of erythrocytes and presumably provide an accurate assessment of reticulocyte percentage.

Since reticulocyte maturation time is not measured, some assumption concerning this value must be made when the reticulocyte count is used as an indicator of the normality of the RBC life span. The common tendency to base the diagnosis of increased RBC turnover on the finding of a reticulocyte count that exceeds the upper limit of normal indicates that the clinician tacitly assumes a "normal" maturation time in the patient with suspected hemolysis. However, a previous report suggested that reticulocyte maturation time may deviate from normal, e.g., subjects with severe hemolysis may have a prolonged maturation time that causes the reticulocyte percentage to overestimate the severity of the hemolytic process [21].

The present study appears to be the first to make a series of near concurrent measurements of reticulocyte percentage and RBC life span under relatively steady-state conditions in patients with mild hemolytic anemia. These measurements provided a unique opportunity to estimate RBC maturation time in the mild hemolytic state associated with RBV therapy. If maturation times were "normal" in RBV-treated subjects, then the RBC survival calculated from the reticulocyte fraction and the normal maturation time of 1.3 days [eq. (5)] should equal the true RBC survival (determined from CO). However, as shown in Fig. 3, in 26 of 29 observations, the reticulocyte-based RBC survival was greater than that calculated from CO. The failure of the reticulocyte count to rise inversely with the decrease in RBC survival presumably reflects an abnormally short reticulocyte maturation time in the RBV-treated subjects. For the RBC life span calculated from the reticulocyte count to agree with the CO measurement, reticulocyte maturation time would have to have been about 0.85 days rather than the normal value of 1.30 days. It is not clear to what extent this putative reduction in reticulocyte maturation time, which contrasts with previous work suggesting prolonged maturation times in severe hemolytic states, is peculiar to RBV-associated anemia.

It should be noted that ineffective erythropoiesis results in release of CO. Thus, the possibility cannot be excluded that the discrepancy between RBC survivals determined from reticulocyte percentage versus CO is explained by RBV-induced ineffective erythropoiesis that caused CO to overestimate the turnover of peripheral RBCs. This seems unlikely given that the deleterious effect of RBV on RBCs is thought to result from the accumulation of this drug in circulating erythrocytes, which causes membrane changes resulting in premature sequestration [9].

CONCLUSIONS

This study demonstrates that RBV-induced anemia consistently is associated with reduced RBC survival as determined from breath CO measurements and this reduced survival frequently is not associated with an elevated reticulocyte count. In addition, we believe this study also illustrates the potential clinical utility of alveolar CO measurements in the assessment of RBC lifespan. This simple, non-invasive test requires the administration of no exogenous material, provides quantitative information on RBC survival within 24 hr of ordering the test (as opposed to weeks with the RBC-tagging methods), and allows for repeated survival measurements at daily intervals, if so desired. Unmanipulated breath and atmospheric samples are injected directly into the gas chromatograph, and the analytical time is only about 1 min. A drawback of this methodology is that it is not applicable to smokers or subjects with impaired pulmonary function.

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