Relationship Between GHb Concentration and Erythrocyte Survival Determined From Breath Carbon Monoxide Concentration

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OBJECTIVE — Subjects with decreased erythrocyte survival have an unusually low GHb percentage. The goal of this study was to determine whether hyperglycemia, as reflected by GHb percentage, is associated with decreased erythrocyte survival.

RESEARCH DESIGN AND METHODS — Erythrocyte survival was quantitated in 23 subjects with type 2 diabetes, and these values were correlated with the subjects' GHb percentage. Erythrocyte survival was determined from the difference between the subjects' alveolar carbon monoxide (CO) concentration and atmospheric CO concentration. Reticulocyte counts were obtained in 16 subjects.

RESULTS — Although the vast majority of the subjects had erythrocyte life spans that fell within the normal range (123 ± 23 days), there was a highly significant inverse correlation ($r = -0.66$, $P < 0.01$) between life span and GHb percentage, with an average decline in life span of 6.9 days for each 1% rise in GHb. The reticulocyte count inversely correlated with erythrocyte life span ($r = -0.77$, $P < 0.01$).

CONCLUSIONS — Hyperglycemia, as evidenced by high GHb percentage, is associated with an appreciable decrease in erythrocyte life span. Because GHb appears to be formed over the lifetime of the erythrocyte, this decreased erythrocyte survival suggests that high GHb percentages may systematically underestimate the true degree of hyperglycemia.

Diabetes Care 27:931–935, 2004

Measurements of GHb and its predominant component, HbA_{1c}, have become the routine means of assessing blood glucose control in patients with diabetes. It is well accepted that glucose combines with Hb via a slow irreversible nonenzymatic reaction, the rate of which is determined by the serum glucose concentration (1,2). Thus, GHb percentage can be used as a time-averaged index of the blood glucose concentration to which the Hb has been exposed. However, unusually low GHb percentages are observed in subjects with hemolytic anemia (3–7). Thus, GHb concentration also is a function of the duration of exposure of Hb to blood glucose, i.e., the life span of the erythrocyte. Of particular interest was a study by Peterson et al. (8), which showed that the life span of ^{51}Cr-labeled erythrocytes increased in each of seven subjects when their poorly controlled diabetes was brought under control. This observation suggests that hyperglycemia reduces erythrocyte survival, a phenomenon that would cause high GHb levels to consistently underestimate the severity of hyperglycemia. The small sample size (seven subjects) in the study by Peterson et al. (8) presumably is attributable to the time-consuming and cumbersome nature of ^{51}Cr labeling studies, which require repeated blood samplings over a multiweek period to obtain a single erythrocyte survival measurement. We recently described a simple and rapid technique to quantitate erythrocyte survival based on measurements of the concentration of carbon monoxide (CO) in expired air (9,10). In the present study, this technique was used to study the relationship between GHb percentage and erythrocyte survival.

RESEARCH DESIGN AND METHODS

Measurements of erythrocyte survival were obtained on 25 occasions in 23 nonsmoking subjects with type 2 diabetes. The subjects were ambulatory men aged 40–77 years. All subjects had normal complete blood counts and serum bilirubin, alanine aminotransferase, and bicarbonate values. Serum creatinine was within normal limits in 21 of the 23 subjects, whereas 2 subjects had values between 1.3 and 1.5 mg/dl. None of the subjects had clinical evidence of pulmonary disease, heart failure, or dehydration, and none were alcoholic. Although the subjects were taking multiple medications, none were receiving drugs known to be prominent inducers of cytochromes, such as barbiturates, rifampin, or dilantin. The study was approved by the institutional review board of the Minneapolis Veterans Affairs Medical Center 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 used to measure alveolar CO have been described in detail in a previous publication (10). In brief, immediately upon awakening in the morning,

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Received for publication 3 September 2003 and accepted in revised form 2 December 2003.

See accompanying editorial, p. 1013.

subjects closed their nares with a nose pinch, inhaled normally, 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-s 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. After collection of a second, duplicate breath sample, the subjects aspirated an atmospheric sample from the bedroom into a 20 ml syringe, and the syringe 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 carbon dioxide (CO_2) concentration via an infrared analyzer (CAPSTAR-100; CWE, Ardmore, PA). The rare sample that contained <5% CO_2 was discarded. Concentrations of CO were determined by gas chromatography using an instrument equipped with a 400-μl gas sampling valve, a column (6 ft × 0.125 in) packed with molecular sieve 5A, and a reduction detector (RGD2; Trace Analytical, 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. The means of the results of the duplicate CO measurements of alveolar air were determined for use in subsequent calculations. The precision of the CO assay was 0.3% (coefficient of variation) at a CO concentration of 5.2 ppm.

Hematologic measurements

Hb values and reticulocyte percentages were determined via a Coulter GEN-S apparatus. Blood was analyzed for reticulocyte percentage on 16 of the 25 occasions that CO measurements were obtained. A boronate affinity high-performance liquid chromatography method using a Variant II (Bio-Rad) system was used to measure total GHb, and these values were then adjusted to national standards using calibrators certified by the National Glycohemoglobin Standardization Program.

Calculations

Survival of erythrocytes based on CO measurements was calculated from (9):

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

where 22,400 is the milliliters of CO per mole and 4 is the moles of CO released 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 parts per million); 64,400 is the molecular weight of Hb; and 1,440 is minutes per day. Because blood volume and resting alveolar ventilation vary with body weight and both have a roughly similar magnitude if blood volume is expressed as milliliters and alveolar ventilation as milliliters per minute, these two values cancel out in the above equation. When [Hb] is in units of grams per milliliter and endogenous P_{CO} is parts per million, Eq. 1 reduces to the simple expression:

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

The reproducibility of this technique has been studied in nine healthy subjects (11). Duplicate computed erythrocyte survivals varied by 3.0 ± 3.0% (mean ± SD), and computed measurements made on samples obtained on consecutive days varied by 10 ± 4.0%.

Normalization of observed glycohemoglobin concentration for erythrocyte survival

Assuming glycation to be linear over the life of an erythrocyte, the predicted GHb percentage (if a subject's erythrocytes had a normal life span) was calculated from:

$$\text{predicted GHb} = (\text{observed GHb}) \times (123 \text{ days}/\text{observed erythrocyte survival}) \quad (3)$$

where 123 days is the mean erythrocyte survival previously observed in 40 healthy control subjects using the breath CO technique used in the present study (10). All results are expressed as mean ± 1 SD.

RESULTS — Figure 1 depicts the erythrocyte life span (calculated from CO measurements) against the GHb concentration for the 23 diabetic subjects. The mean erythrocyte survival of the diabetic subjects was 112 ± 25 days, compared with the previously determined normal mean survival of 123 ± 23 days (10). Two subjects had erythrocyte survivals that fell just below the normal range, whereas the remainder were normal. There was no significant correlation ($r = 0.08$) between the atmospheric concentration of CO and the calculated erythrocyte survival, indicating that varying atmospheric exposure to CO did not systematically influence the survival calculations. The total GHb percentage of the subjects with diabetes ranged from 6.1 to 14.8%, with a mean value of 8.8 ± 2.5%. The reference range for our laboratory is 4.7–6.3%. As shown in Fig. 1, a highly significant inverse correlation existed between GHb and erythrocyte survival ($r = -0.66$, $P < 0.01$). The slope of this plot indicated an average decline in erythrocyte survival of 6.9 days for each 1% increase in GHb.

Figure 2 shows a plot of erythrocyte survival versus reticulocyte count in 16 of the subjects. The reticulocyte percentage averaged 1.07 ± 0.57% compared with the mean of 1.08% for healthy subjects. A strong inverse correlation ($r = -0.77$, $P < 0.01$) was observed between reticulocyte count and erythrocyte survival.

CONCLUSIONS — Although the concept that hyperglycemia reduces erythrocyte survival is not original (9), testing of this possibility in a large group of diabetic patients has been limited by the complexity of the techniques available to measure erythrocyte life span. The

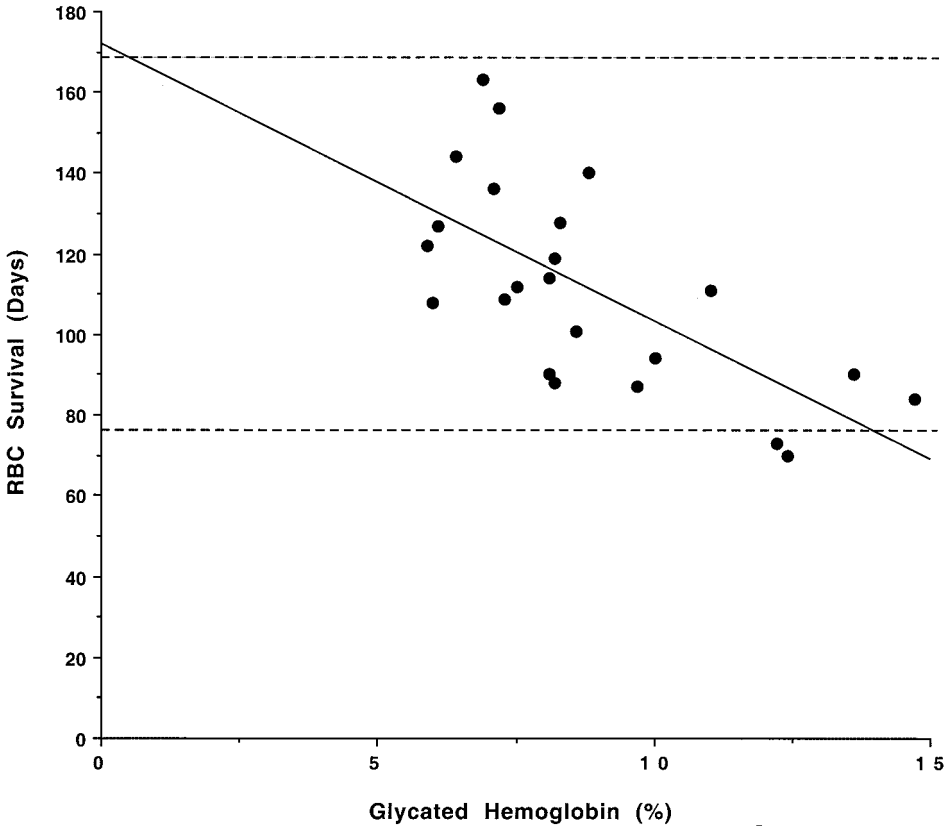

Figure 1—*Plot of erythrocyte (red blood cell [RBC]) survival versus GHb percentage of 23 diabetic subjects. A highly significant inverse correlation* ($r = -0.66$, $P < 0.01$) *was observed. The dotted lines represent the limits of normal for erythrocyte survival determined in a previous study (10).*

"gold standard" technique for quantitating erythrocyte survival involves administration of a label that is irreversibly incorporated into erythrocyte precursors in the bone marrow (12–14). Survival measurements require that the disappearance of tagged cells from the circulation be monitored over a period exceeding 120 days, a requirement that limits both the clinical and research applicability of this technique. The only erythrocyte survival measurement used to any extent in clinical practice involves the labeling of circulating erythrocytes (usually with ^{51}Cr) and then following the disappearance of the label from the circulation. Inaccuracies resulting from elution of the radioactive label and the requirement for multiple venesections over a multiweek period have limited the use of this methodology.

In the present study, we used measurements of breath CO concentration to quantitate erythrocyte survival. All CO generated in the body is thought to be derived from the α-methene carbon of heme, which is stoichiometrically released as CO when heme is converted to bilirubin (15). Hb catabolism represents the vast majority of heme turnover, and measurement of expired CO (corrected for environmental CO) thus provides a quantitative measure of erythrocyte turnover. Early studies using CO to assess erythrocyte life span used complicated rebreathing techniques to differentiate endogenous from atmospheric CO (16,17). We have described and validated a simple, rapid, and noninvasive technique in which erythrocyte life span is determined from the difference between the CO concentrations of samples of alveolar and atmospheric air (9,10), and this technique was used in the present study to quantitate erythrocyte survival in subjects with diabetes.

Although normal erythrocyte survival is commonly considered to be 120 days, several different measurement techniques have shown appreciable normal variability around a mean of ~120 days. We found a mean erythrocyte survival of 123 ± 23 days (1 SD) in a study of 40 healthy control subjects using the CO technique used in the present investigation, a variability comparable to that observed with more complicated methodology (16,17).

The 23 subjects with type 2 diabetes had a mean erythrocyte survival of 112 ± 25 days, and only 2 subjects had survival values that were slightly outside the normal range. Nevertheless, a highly statistically significant inverse correlation ($r = -0.67$, $P < 0.01$) existed between erythrocyte life span and GHb percent (Fig. 1), a finding that agrees with the observation of Peterson et al. (8) that erythrocyte survival measured with ^{51}Cr increased with control of hyperglycemia. This correlation suggests that the observed individual differences in survival, although largely falling within the normal range, represented true differences rather than simply "noise" arising from inaccuracy of the breath CO technique.

It should be noted that the CO technique used to measure erythrocyte life span assumes normal ventilation, blood volume, and cytochrome turnover, and major deviations from normality could influence the erythrocyte survival measurements. However, the erythrocyte life spans of subjects with the highest GHbs were ~40% less than those with the lowest GHbs. Very severe abnormalities of ventilation and/or blood volume would be required to produce a computed survival difference of 40%, and no such abnormalities were present in any of the subjects. The possibility that unusually rapid turnover of cytochromes released excessive CO is also remote. None of the subjects had clinical or laboratory evidence of liver disease, and none were alcoholic. In addition, none of the subjects was taking drugs known to be major inducers of cytochromes, such as barbiturates or dilantin. The finding that the reticulocyte counts of the diabetic subjects had a highly significant inverse correlation ($r = -0.77$, $P < 0.01$) with the erythrocyte survival measurements (Fig. 2) provides strong confirmatory evidence that the observed differences in erythrocyte life span were real and not an artifact of the CO technique. Although our observation that erythrocyte life span correlates with GHb percentage is in accordance with that of a previous study of seven subjects whose erythrocyte survival was assessed via ^{51}Cr tagging (8), it would be

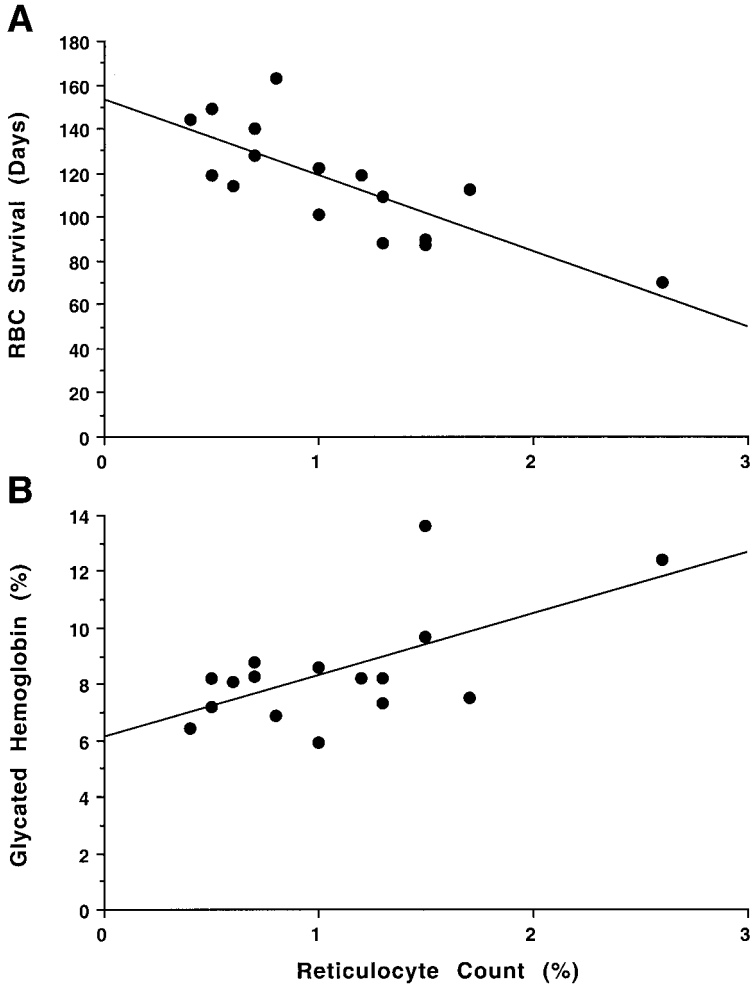

Figure 2—*A: Plot of erythrocyte survival versus reticulocyte count of 16 diabetic subjects. RBC, red blood cell. A significant* ($r = -0.77$, $P < 0.01$) *inverse correlation was observed.* B*: Plot of GHb versus reticulocyte count in the same 16 subjects. A significant* ($P < 0.02$) *positive correlation was observed.*

useful to confirm this correlation in a larger group of diabetic subjects using ^{51}Cr labeling methodology.

The correlation between erythrocyte life span and GHb percentage suggests that high blood glucose levels induce alterations in erythrocytes that result in their more rapid removal from the circulation. Although the mechanism is speculative, it has been demonstrated that erythrocyte membrane fluidity is decreased in diabetic individuals, an alteration that could result in excessively rapid destruction of these cells (18). It is also known that glycation alters the function of Hb (e.g., increases oxygen affinity) and increases erythrocyte adherence to endothelial cells (19), and it seems possible that high levels of GHb increase erythrocyte turnover.

On average, erythrocyte life span decreased by ~6.9 days for every 1.0% increase in GHb. The four subjects who had GHb percentages >12 had a mean erythrocyte survival of 81 days, ~65% of the normal mean of 123 days. This finding may have clinical implications because such a diminished erythrocyte survival would reduce the GHb percentage that would be observed if the cells survived normally. The precise time course of glycation of erythrocytes has not been clearly established. However, older erythrocytes are more highly glycated than younger cells (20–22), and it seems likely that glycation occurs over the life of the erythrocyte. Assuming that glycation occurs at a linear rate over the erythrocyte life span, GHb values of 12–15% would be increased by ~50% when corrected for a normal erythrocyte life span (see Eq. 3), i.e., these values would have been ~18 to ~22.5%, respectively, if the hyperglycemia had not induced a 35% reduction in erythrocyte life span. Such a tendency for high GHb levels to underestimate the true degree of hyperglycemia could explain the observation that the rate of progression of retinopathy of diabetic subjects rises out of proportion to the apparent abnormality of their GHb percentage (23).

Measurements of GHb are widely used to assess the efficacy of various forms of therapy on glycemic control and to predict the expected incidence of various complications of hyperglycemia. The validity of conclusions drawn from GHb measurements is a function of the fidelity with which GHb percentages reflect blood glucose levels. One factor other than blood glucose concentration that can influence GHb percentage is individual differences in erythrocyte survival. Such survival differences could simply reflect the inherent variability of erythrocyte life span that has been observed in the healthy population. In addition, erythrocyte life span could be altered by conditions independent of the diabetic process or therapies used to treat diabetes or associated diseases. Finally, the present study suggests that there is a hyperglycemia-related decrease in erythrocyte survival that results in a progressively greater underestimation of the severity of the hyperglycemia the higher the GHb percentage. Confirmation of this concept will require study of a large cohort of subjects whose blood glucose concentration is known to be relatively stable over the lifetime of the erythrocyte.

Acknowledgments— This study was supported by General Medical Research funds from the U.S. Department of Veteran Affairs.

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