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Red blood cell volume can be independently determined *in vivo* in humans using red cells labeled at different densities of biotin

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Abstract

BACKGROUND—Anemia is a serious problem in critically ill neonates. To investigate the pathophysiology of anemia and responses to red blood cell (RBC) transfusions and erythropoietin therapy, repeated measurement of red cell volume (RCV) and blood volume are useful. To extend our previous sheep study in which RBCs were labeled at four different biotin densities, we assessed the validity of this multidensity method for *in vivo* measurement of circulating RCV in humans.

STUDY DESIGN AND METHODS—In eight healthy adults, autologous RBCs were biotinylated at each of four biotin densities (6, 18, 54, and 162 µg biotinylation reagent per mL RBC), mixed, and infused intravenously; blood was sampled at 10, 20, and 60 minutes. At each time, RCV was calculated from dilution of individual RBC populations enumerated by flow cytometry. RCV measurements from the population of RBCs biotinylated at 6 µg/mL were chosen as the reference values because this density had been previously validated against the ⁵¹Cr method *in vitro* and *in vivo* in humans.

RESULTS—Values for RCVs were not significantly different among the four densities of biotinylated RBCs at any of the three time points and did not change over 60 minutes.

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Authorship Contributions

Contribution: D.M.M. is the Biotin Core Lab Director and interpreted results and prepared manuscript for publication. N.I.M. developed the *in vitro* labeling of RBCs with multiple densities, designed data analysis spreadsheets, and assisted in manuscript preparation. S.Z. prepared RBCs for flow analysis, performed data analysis, and assisted in manuscript preparation. L.F.B. interpreted statistical results and assisted in manuscript preparation. M.B.Z. performed statistical analysis of data and assisted in manuscript preparation. R.G.S. interpreted results and assisted in manuscript preparation. R.L.S. performed *in vivo* labeling of RBCs with infusion and post transfusion sampling and assisted in manuscript preparation. D.N. performed *in vivo* labeling assisted in manuscript preparation. G.C. served as clinical coordinator, assured compliance with regulatory requirements, obtained informed consent, and assisted in manuscript preparation. J.A.W. is the overall Program Project Director, directed the biotinylation of RBCs, interpreted results, and assisted in manuscript preparation.

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CONCLUSION—These studies provide evidence that four densities of biotinylated RBCs can be used *in vivo* for simultaneous, independent, accurate measurements of RCV in humans. We speculate that this method will also be useful for repeated measurement of RCV and blood volume in infants and other patient populations in whom radioactive labels should be avoided.

INTRODUCTION

Transfusion medicine and hematology studies of red blood cell (RBC) kinetics, including the measurement of RBC volume (RCV) in infants, children, and pregnant women and their fetuses have been hampered by several problems. In particular, there have been few studies in the fetus and extremely preterm (VLBW), critically ill infants in whom RCV has been directly measured. Radioisotopes, most commonly chromium 51 (^{51}Cr), employed for labeling either autologous or allogeneic RBCs had raised sufficient concerns about radiation exposure in fetuses and infants¹ that use was abandoned by the late 1960s.² Methods that do not utilize radioisotopes have several technical limitations including cost, feasibility, and sensitivity. For example, production of the stable chromium isotope, ^{50}Cr , requires an expensive particle accelerator³. Moreover, like ^{51}Cr , measurement of RCV using ^{50}Cr is subject to error from the variable elution of Cr from the RBC^{4,5}. Although RCV methods based on RBC antigen mismatches or the dilution of HbF by HbA⁶ are sufficiently sensitive for performing RCV measurements when allogeneic donor (but not autologous) blood is used, application of these methods to sequential studies of RCV is problematic.^{7,8}

Thus, a reliable and feasible method for measuring RCV that does not use radioactive labels and allows multiple measurements of either autologous or allogeneic blood would be of value in defining the physiology, pathophysiology, and response to treatment of a variety of conditions in vulnerable patient populations, including pregnant women, fetuses, and infants. Successful validation of the biotin method is important because this method has several notable advantages over currently validated methods. The biotin method does not expose study subjects to radiation and, although the method requires only a few microliters of blood, it is sufficiently accurate and sensitive for use in fetuses, VLBW neonates, and adults^{9,10}. Moreover, the biotin method is practical because 1) the required biotinylation reagents are inexpensive and commercially available, 2) the required instruments (a flow cytometer and sterile hood) are available in most medical center clinical laboratories, and 3) technician time required for analysis is modest.^{10,11}

Multiple distinct populations of biotinylated RBCs can be reproducibly and predictably prepared, mixed, and individually enumerated¹². This permits the repeated determination of RCV (and calculation of blood volume) in the same subject; such measurements are particularly important in clinical situations associated with factors such as ongoing erythropoiesis, somatic growth, and RBC transfusions. In a previous *in vitro* study, we employed five discrete populations of RBCs that differed in biotinylation density to validate this approach using sheep and human RBCs⁸. Subsequently, we refined this approach; using sheep RBCs biotinylated at four discrete densities, we measured simultaneously RCV *in vivo* and observed no differences among the four densities of biotinylated RBCs at multiple post-infusion sampling times¹³. However, we did observe a significant decline in the percent of biotinylated RBCs in the circulation during the first 60 minutes post-transfusion. This decline is consistent with equilibration of circulating RBCs with a more slowly mixing pool of RBCs in the sheep spleen, as has been suggested by previous studies^{14,15}.

Accordingly, the primary objective of the current study was to assess the accuracy of RCV determined simultaneously and independently *in vivo* in humans using four distinct densities of biotinylated RBCs; our secondary objective was to determine whether a similar equilibration phenomena was observable in healthy adults in the hour following transfusion.

Our hypotheses were 1) that RCV determined using the three highest densities would agree with the RCV determined using RBCs labeled at the lowest density and 2) that RCV determined at three times over the 60 minutes following infusion of the biotinylated RBCs would not differ.

MATERIALS AND METHODS

Human studies

All studies were approved by the University of Iowa Committee on Research on Human Subjects (study performance site) and the Institutional Review Board of the University of Arkansas for Medical Sciences (study analysis site). Informed consent was obtained from each subject.

Study Population

Inclusion criteria included the following: 1) 18–65 years of age; 2) weight > 50 kg; 3) hemoglobin ≥ 125 g/L; and 4) negative direct antibody test (DAT). Exclusion criteria included the following: 1) presence of an active chronic illness; 2) consumption of biotin supplements or raw eggs; 3) blood donation in the previous 8 weeks; 4) blood loss in the previous 8 weeks due to epistaxis, gastrointestinal bleeding, trauma, significant diagnostic phlebotomy (>30 mL), or other bleeding deemed likely to be hematologically significant by the study physician; 5) premenopausal women to avoid the complication of intervening menstrual blood loss; and 6) treatment with antibiotics in the week prior to initiating study participation.

Eight healthy adults (5 women) participated in this study. Ages ranged from 23 to 62 years. Body mass ranged from 57.2 to 115.2 kg.

Summary of experimental design

In each subject at each of three post-transfusion times (10, 20 and 60 minutes), circulating RCV was determined *in vivo* simultaneously and independently by dilution of autologous RBCs labeled at four discrete densities of biotin.

Collection of autologous blood

A 21 gauge $\times \frac{3}{4}$ in push button blood collection set (Becton, Dickinson, and Co., Franklin Lakes, NJ) was placed percutaneously in the antecubital vein, and venous blood was collected to provide a baseline complete blood count, DAT, haptoglobin, and plasma free hemoglobin. After verification that the blood concentration of hemoglobin ≥ 12.5 gm/dL and was negative for the DAT, 250 mL of autologous blood was collected for biotinylation and for use in the standard curve (see below). A Blood-Pack Unit with 16 gauge ultra-thin wall needle and blood collection bag designed for collection of 250 mL was used for blood collection; these units contain 35 mL of CPD (citrate-phosphate-dextrose preservative) and were manufactured at the time of purchase by Baxter Healthcare Corp., Deerfield, MA (now manufactured by Fenwal Inc, Lake Zurich, IL). The needle was placed percutaneously in the antecubital vein and 250 mL (260 g) of blood was collected; adequacy of sample volume was judged gravimetrically using a scale (Ohaus, Pine Brook, NJ).

Biotinylation of RBCs

Although there is a variety of biotinylating reagents available commercially, there is no biotinylation reagent that is specifically approved for human use. Because the biotin reaction with the amino group produces the same biotinylation of the epsilon amino group of lysine seen in biotin-dependent carboxylases and biotinylated histones and because biotinylation is

not known to generate toxic byproducts, we speculated that no toxicity would be observed with the use of biotinylated RBC in human subjects. Notwithstanding, we have obtained and annually renewed an Investigator New Drug approval from the FDA for these human studies. Under the approved IND protocol, we filter sterilized the biotinylation reagent using a 0.22 μm filter.

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To examine the biotinylation reaction products for unexpected toxicity, cytotoxicity was assessed by an outside reference laboratory (HemoGenix, Colorado Springs, CO 80907, <http://www.hemogenix.com/>). The assay system (HALO-96 Suspension Expansion) cultured human bone marrow mononuclear cells (in replicates of 8) at a final cell concentration of 10,000 cells/well in 96-well plates. Three adult blood samples were biotinylated at the lowest and highest biotin densities. 100 μL of culture reagent was added to 11 μL of each wash of the biotinylated RBCs. Wash fluid not used for washing the biotinylated RBCs and no addition of served as controls. After 5 days in culture, ATP concentration – an indicator of proliferation or cytotoxicity – was measured. No statistically significant decreases in ATP concentration occurred, indicating that the wash fluid from the biotinylated RBCs had no effect on either the proliferation or cytotoxicity status of the target cells. These data provided evidence that even the first wash of the biotinylation reaction with RBCs resulted in no measurable toxicity in a sensitive hematopoietic stem cell assay. In this study of three normal subjects, we also cultured the initial blood sample and the final suspension of biotinylated RBCs contamination; these cultures were negative.

The method for biotinylating RBCs to produce distinct biotinylation densities on the cell surface has been previously described.¹² Briefly, individual aliquots of autologous RBCs were incubated with increasing concentrations of sulfo-succinimido-biotin (Pierce Chemical Co., Rockford, IL). A freshly prepared stock solution of the biotinylation reagent was diluted serially in wash buffer at pH 5.0 to favor the biotinylation reaction over hydrolysis of the reagent; the biotinylation reaction used to produce discrete densities of biotin label on the RBC surface proteins was carried out in wash buffer (pH= 7.4). The wash buffer composition was 11.1 mmol per L glucose, 20 mmol per L sodium bicarbonate, 2.3 mmol per L NaHPO_4 , 1.14 mmol per L Na_2PO_4 , and 154 mmol per L NaCl . This wash buffer is prepared by adding 8 mL 50% Dextrose, 20 mL Sodium Bicarbonate, and 1 mL Sodium Phosphates to 1000 mL Normal Saline; all these components were “USP for injection” grade reagents. The biotinylation reaction was stopped after 30 minutes by washing the RBCs two times in five fold greater volumes of wash buffer; this also removed the remaining biotinylating reagent as well as any reaction and hydrolysis byproducts. These washed biotinylated RBCs were passed through an 18 μm filter (Hemo-Nate Syringe Filter, Utah Medical Products, Inc, Midvale, UT). The mixture of biotin-labeled RBCs for each subject was cultured for bacterial contamination; these cultures were negative.

Infusion and sampling

Immediately prior to infusion of biotinylated RBCs, a catheter (Cathlon 18 or 20 gauge $\times$ 1.16 in, Smiths Medical MD, Inc., London, England) was placed percutaneously in the antecubital vein; this catheter was used both for infusion of biotinylated RBCs and for obtaining post-transfusion blood samples. RBCs biotinylated at the four discrete densities were mixed together and reinfused as a bolus over 3 to 4 minutes; time at the end of the

bolus infusion was designated time zero. For each density population ($j = 1, 2, 3$, or 4), the number of biotinylated RBCs infused ${}^j\text{RBC}_i$ was precisely determined from total red cell count (RCC) of the mixture, the mass of the transfused RBC mixture determined gravimetrically by mass difference of the infusion syringe (W_i), the proportion of each population in the mixture (f_j) determined gravimetrically, and the specific gravity (SG_i) of the infused mixture using the following equation:

$${}^j\text{RBC}_i = [\text{RCC}_i \text{ (\#/mL)} * W_i \text{ (g)} * f_j] / [SG_i \text{ (g/mL)}]$$

The amounts of each population of biotinylated RBCs infused were chosen to produce a final percentage of about 2% of total RBCs in circulation. The RCC (total number of RBCs per μL) for the unmixed populations of biotinylated RBCs and the venous blood samples (see below) were measured using a Sysmex XT-2000iV (Sysmex Corp, Kobe, JP).

Quantitation of proportion of biotinylated RBCs

After infusion of the biotinylated RBCs, the intravenous access tubing was flushed with 5 mL of normal saline. Subsequent venous blood samples were drawn and exact sample times were recorded relative to time zero. For each sample of venous blood, the concentration of each distinct population of biotinylated RBCs was determined by flow cytometric enumeration as previously described.¹³ In this study, each population of biotinylated RBCs appeared as a distinct peak on the fluorescent intensity histogram obtained from flow cytometry (see Figure 1). The number of biotin labels per RBC (and in turn, the degree of separation between histogram peaks) is determined by the concentration of sulfo-succinimido-biotin reagent. In this study, we observed the same linear relationship between fluorescent intensity per RBC and mass of biotinylation reagent per mL of RBCs previously reported.⁸ A reagent concentration of 6 μg per mL of RBC (18 nmol/mL of RBC) was chosen as the lowest density because this density still provided complete separation from the unlabeled RBCs (Figure 1). To avoid any overlap between the adjacent histogram peaks (Figure 1) while minimizing the increment in reagent concentration and thus maximizing the number of RBC populations studied, the biotinylation reagent concentration was increased in three-fold increments to 18, 54, and 162 $\mu\text{g}/\text{mL}$ of RBCs (53, 158, and 474 nmol/mL of RBCs).

Calculation of RCV and blood volume

At each blood sampling time, RCV was calculated independently for each of the j biotinylation densities by application of the formula derived from the dilution principle described previously¹³ as follows:

$$\text{RCV}_j = {}^j\text{RBC}_i * \text{HCT} / (\text{RCC} * \%B_j)$$

where ${}^j\text{RBC}_i$ is defined above, HCT is the venous hematocrit of the recipient, RCC is the red cell count expressed per mL of the venous blood of the recipient, and $\%B_j$ is the percentage of biotinylated RBCs for the j th density in the venous blood of the recipient. The percentage of biotinylated RBCs in each distinct population at each time was obtained from the histograms of flow cytometric enumeration of the RBC density peaks. The percentage of biotinylated RBCs was corrected against a standard curve prepared as previously described.^{9-12,16,17} The slope of the standard curve was consistently within 10% of unity, suggesting that there was little error in the enumeration of the populations of biotinylated RBCs. Blood volume was calculated as RCV/HCT . The blood volume calculated will be underestimated by the extent to which these venous HCT overestimate the total body hematocrit.^{18,19}

Statistical methods

In humans, we previously validated quantitation of RCV using RBCs labeled at a single low density of biotin⁹ or at two low densities of biotin¹⁰ against the ⁵¹Cr reference method. In the primary comparison of the present study, we assessed the validity of RCV determined simultaneously, but independently, at a given time using RBCs biotinylated at densities 18, 54, and 162 µg/mL; RCV from the lowest density of biotinylated RBCs (6 µg/mL) was used as the reference RCV value. In a secondary comparison, we compared RCVs determined from RBCs biotinylated at all of the densities among the three time points. The following statistical approaches were used to assess agreement among the RCVs determined from the various populations of biotinylated RBCs and among the RCVs determined at 10, 20, and 60 minutes:

1. RCV values from the RBCs labeled at the higher biotin densities (18, 54, and 162 µg/mL) were plotted against the RCV values for the 6 µg/mL density. Each linear regression was examined for statistical difference from the line of identity by assessing whether a) the 95% confidence intervals for the slope included a slope of 1.0 and b) whether the 95% confidence intervals for the y-axis intercept included the origin.
2. In addition to comparison of the RCV values, an analysis based on the ratio of the RCV values for the three highest densities relative to the 6 µg/mL density was also performed. This was done because RCVs are usually compared relative to one another. Using this approach, a linear mixed model analysis for repeated measures was used to test for differences among the mean RCV ratios, with density, time, and density*time interaction as the fixed effects. In addition to estimating the fixed effects in the mixed model, this method of analysis allows selection of the covariance structure that best fits the relationship of the RCVs for the different densities and at the different times measured in the same subject (i.e., repeated measures). From the mean and standard deviation estimates of the fitted linear mixed model, the 95% limits of agreement for the mean ratio (mean $\pm$ 1.96 SD) were computed.²⁰ This interval defines the range within which lie most of the individual RCV ratios of the higher biotin density (18, 54, or 162 µg/mL) to the reference density of 6 µg/mL. Bland-Altman plots of the observed RCV ratio for a given density (e.g., ratio of 18 to 6 against the RCV value from density 6 for the same individual at the same time) were constructed to display the distribution of ratios. The computed 95% limits of agreement are also displayed.²⁰

RESULTS

Complete RCV values for each of the eight subjects at all three time points for all four biotin densities are provided in the Table 1. Examination of each the four biotinylation densities indicates 1) that, over the hour post-infusion, the RCVs determined from each the four biotinylation densities agreed well with each other at all three times and 2) that RCV did not change over the one hour study period. RCV values calculated from the dilution of RBCs biotinylated at any given density did not change significantly between 10 and 60 minutes. For the group of all subjects, no significant change with time was detected by the linear mixed model analysis for repeated measures discussed below.

Using the RCV values for the 6 µg/mL RBCs and all three time points, the mean RCV $\pm$ 1 SD was = 22.9 $\pm$ 2.5 mL/kg body weight; the range 19.2 to 26.4 mL/kg. The mean blood volume for the study subjects was 58.5 $\pm$ 5.1 mL/kg; the range was 50.8 to 66.7 mL/kg.

The single notable exception was Subject 4 (Table 1). The RBCs biotinylated at 162 µg/mL disappeared rapidly; as a consequence, the RCVs calculated from the 162 µg/mL density for

Subject 4 increased rapidly and were unphysiologically large. A repeat study in the same subject 10 months later exhibited the same rapid, selective removal of the RBCs biotinylated at 162 $\mu\text{g/mL}$, which again contrasted strikingly with the nearly 100% survival between 10 and 60 minutes of RBCs biotinylated at each of the three lesser densities; likewise, there was good agreement among the RCVs calculated from the lesser densities. Because these results suggest a consistent biological characteristic unique to this subject (rather than an analytical artifact), the 162 $\mu\text{g/mL}$ density data for Subject 4 were excluded from all subsequent analyses.

Because there was no significant difference between RCVs at 10, 20, and 60 minutes for each subject, we used all time points for the regression comparison of each of the higher densities (18, 54, and 162 $\mu\text{g/mL}$) against the “gold standard” reference density (6 $\mu\text{g/mL}$). Figure 2 Panels A–C depict those regression comparisons and tabulate the regression parameters. For densities 18 and 54, the regression lines are not significantly different from the line of identity as judged in two ways: 1) the confidence intervals (CI) for the slopes incorporate 1; and 2) the CIs for y intercepts incorporate 0. For density 162 $\mu\text{g/mL}$, the upper limit of the 95% CI for the slope was 0.98 and thus failed to incorporate 1; in addition, the lower limit of the 95% CI for the y intercept was 64 and thus failed to incorporate 0. This result is consistent with an accelerated artifactual removal of RBCs biotinylated at 162 $\mu\text{g/mL}$. Although the difference is statistically significant, the artifact introduced is small and likely of little clinical significance. The mean RCV overestimate using 162 $\mu\text{g/mL}$ RBCs was only 1.3% (SD = 2.8%) greater than the RCV obtained using density 6 $\mu\text{g/mL}$.

To determine whether there was a detectable effect of biotin density on RCV when all data were considered, we performed a linear mixed model analysis for repeated measures on the ratios of the RCV values from RBCs biotinylated at the higher densities (18, 54, and 162 $\mu\text{g/mL}$) to RCV values for 6 $\mu\text{g/mL}$. Table 2 gives the mean RCV ratios to density 6 $\mu\text{g/mL}$ for densities 18, 54, and 162 $\mu\text{g/mL}$ at each time. There was no significant density*time interaction ($p=0.59$), and there was no significant difference in mean RCV ratios between any of the three time points ($p=0.50$) or between the three density ratios ($p=0.21$). The overall mean RCV ratio for the three higher densities and the three time points was 1.004 (95% CI: 0.990, 1.018).

Using the mean and standard deviation estimates obtained from the fitted linear mixed model, the 95% limits of agreement for the ratio of RCV values for each density to the reference density were computed. For densities 18, 54, and 162 $\mu\text{g/mL}$, the Bland-Altman plots depicting these limits, the mean RCV ratio, and the RCV ratios for each subject are presented in Figure 3A–C. Based on this analysis, 95% of the RCVs determined using RBCs biotinylated at biotin density 18 $\mu\text{g/mL}$ are predicted to be within -5.0% to $+4.2\%$ of the RCV measured using the reference biotin density of 6 $\mu\text{g/mL}$. A similar range is predicted for RCV ratios obtained using RBCs biotinylated at biotin density 54 $\mu\text{g/mL}$ and 162 $\mu\text{g/mL}$ (-5.1% to $+6.2\%$ and -4.7% to $+6.8\%$, respectively). Thus, RBCs labeled at any of the four densities will give equivalent RCV results in most adults; the notable exceptions would be any individuals resembling Subject 4. Accordingly, densities 6, 18, and 54 $\mu\text{g/mL}$ are preferred when only three independent measurement of RCV are dictated by the experimental design.

Discussion

A method to accurately measure circulating RCV (often referred to as red cell mass) without exposure of the patient or research subject to radioactivity is highly desirable. The present study provides evidence that RCVs determined using autologous RBCs labeled at four distinct densities of biotin agree well with one another in healthy adults, thus paving the way

for studies of other groups, including infants, children, and pregnant women and their fetuses. The essential measurement made using this approach is enumeration of biotinylated RBCs as a percent of all RBCs in circulation and the calculation of the total number of RBCs in circulation based on the dilution principle and knowledge of the total number of biotinylated RBCs infused. RCV is calculated from the dilution of biotinylated RBCs and the mean corpuscular volume (or the equivalent ratio of HCT/ RCC), and the blood volume is calculated from the RCV/HCT. The method utilizing biotinylated RBCs does not expose the subject to radiation and has been validated against ^{51}Cr -labeled RBCs (the established standard method) in animal and humans studies using RBCs labeled at a single density of biotin.^{8-10,12,17,21}

To further enhance the utility of the biotin method, we refined the technique for biotin labeling to produce four separate populations of RBCs with increasing biotin density per RBC and validated the use of these RBCs to independently and simultaneously measure RCV *in vitro* and *in vivo* both in sheep²² and human adults. Because the densities are independently enumerated and do not overlap in the histogram (See Figure 1), serial measurements of RCV can be made using each density of biotinylated RBCs without the need to correct for baseline values.¹⁰ Serial RCV measurements have the potential to facilitate quantitative estimates of erythropoiesis despite the intervening events such as hemorrhage, transfusion, and somatic growth that typically confound attempts to quantify erythropoiesis in clinical situations.

Our finding that RBCs labeled with biotin at several densities can be used to accurately and reliably determine RCV is particularly important for measurement of RCV in fetuses, infants, children, and pregnant women. Since the late 1960s, research studies that exposed these groups to radioactivity have been prohibited because of concerns about the potential for harm, regardless of the exposure dose.² During this hiatus, only a handful of infant studies using direct measurements of RCV have been published.^{6,16,23-25} Moreover, no RCV studies in fetuses and only one study utilizing non-radioactive ^{50}Cr in pregnant women²⁶ have been published. We contend that the biotin method is particularly suitable for such studies. Moreover, suitability of cord blood as a source for RBCs for transfusion is an active area of research; because biotin is suitable for labeling RBCs from umbilical cord blood (unpublished data), RCV measurement using autologous cord blood is particularly attractive for such studies.

Several technical points merit emphasis. RCV values, as determined from the four biotin densities, were in close agreement at each of three times. RCV results from all biotin densities did not change significantly over the 60 minutes of measurement, and when extrapolated back to time zero, RBCs biotinylated at each of the four densities yielded a set of RCV values that were not significantly different from the values at 10 minutes. We infer that sampling of blood for measurement of percent of biotin labeled RBCs may be done at any time between 10 and 60 minutes; whether this conclusion can be generalized from fresh autologous RBCs to stored allogeneic RBCs is not specifically addressed by the study presented here. The results from this human study contrast with observations from our studies and those of others in sheep.^{13,27-29} In sheep, a slowly equilibrating pool of RBCs, likely sequestered in the spleen or other reticuloendothelial organs, was observed. In contrast, in the healthy adult subjects of the present study, we found no evidence of circulating RBCs equilibrating with a more slowly mixing pool of RBCs. We infer that, in humans, either the RBC pool in the spleen equilibrates with circulating RBCs before 10 minutes or that the RBC pool in the spleen is smaller than our limit of detection $12 (\pm 5\% \text{ of RCV})$ or both.

Whether this conclusion about healthy adults can be generalized to patients with functional splenic abnormalities is not clear. Examples of such patients would be those with sickle cell disease. Young patients with sickle cell disease may have enlarged spleens capable of sequestering abnormally larger numbers of RBCs and might exhibit RBC mixing phenomena similar to what we¹³ and others^{27–29} have observed in sheep. In contrast, older patients with sickle cell disease are likely to have experienced splenic infarcts substantial enough that the spleen RBC pool is insignificant, thus yielding RBC mixing phenomena similar to healthy adults and rendering timing of blood sampling for RCV measurement unimportant. Similarly, further study is required to determine the effects of splenomegaly, as might be encountered in other hematologic and oncologic disorders, on RCV measurement by this method.

In the present study, we observed one study subject whose RCV data was clearly discrepant with the others, but only at the highest biotin density. Our finding that these discrepant results persisted when the subject was retested provides evidence that the observation was not due to technical error. We speculate that reticuloendothelial system of this subject may have been unusually sensitive to changes induced in RBC surface membrane (e.g., in biotinylated proteins) that resulted in a threshold above which these most densely biotinylated RBCs were rapidly removed. How frequently this phenomenon is likely to be seen in others is uncertain at present based on the small number of subjects studied. For the RBCs biotinylated at density 162 $\mu\text{g/mL}$, we observed a statistically significant but clinically unimportant overestimate of mean RCV for all subjects; we speculate that this phenomenon reflects a subtle, clinically insignificant shortening of survival over the first 60 minutes for the most heavily biotinylated RBCs that resulted in a small overestimate of RCV. Accordingly, we conclude that prudence dictates avoiding use of the 162 $\mu\text{g/mL}$ density biotinylated RBCs unless experimental design dictates independent RCV measurements that can not be made with the three lower densities. Finally, although the present study of RBCs labeled at four biotin densities provide no direct information regarding whether RBCs labeled at even higher biotin densities might also be useful for accurately determining RCV, we speculate that the phenomenon seen in 162 $\mu\text{g/mL}$ density biotinylated RBCs in Subject 4 foreshadows even more rapid removal of RBCs biotinylated at densities greater than 162 $\mu\text{g/mL}$.

The evidence provided here that RCV can be accurately measured in humans using RBCs labeled with multiple distinct densities of biotin is likely to be useful to investigators who wish to perform multiple RCV determinations in studies of human fetuses, infants, children, and pregnant women. Our studies applying RBCs labeled with multiple distinct densities of biotin to measurement of RBC survival (e.g., 24 hour survival post-transfusion recovery and circulating lifespan) are ongoing. We speculate that, as more data for the biotin red cell labeling method accumulate, the biotin method will replace ⁵¹Cr as the reference method of choice for both RCV and red cell survival in many clinical and research settings.

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Figure 1.
Flow cytometry histogram relating number of RBCs enumerated to log of fluorescent intensity for four populations of biotinylated human RBCs demonstrates separation between each population of biotinylated RBCs and from the population of unlabeled RBCs. “6, 18, 54, and 162 $\mu\text{g/mL}$ ” denote the populations of biotinylated RBCs produced by successive three-fold increases in the ratio of the mass of biotinylation reagent to volume of RBCs being biotinylated.

Figure 2A

Figure 2B

Figure 2C

Figure 2. RCV values determined at 10, 20, and 60 minutes from three highest populations of biotinylated RBCs (A: 18 µg/mL, B: 54 µg/mL, and C: 162 µg/mL) plotted against RCV determined from the reference biotin density of 6 µg/mL demonstrated excellent agreement. Two of the three regression lines (18 and 54) were not significantly different from the line of identity.

Figure 3. Individual RCV values from RBCs biotinylated at 18 µg/mL (Panel A), 48 µg/mL (Panel B) and 96 µg/mL (Panel C) agreed well with the lowest biotin density (6 µg/mL). Depicted are ratios to the corresponding RCV value from 12 µg/mL for individual RCV values. Values for all of the three timed blood samples and all eight humans are depicted. Mean ratio (solid line) and the 95% limits of agreement (dashed line) are also depicted.

Table 1

Red cell volumes (RCVs) determined independently and simultaneously from the four biotinylation densities agreed well with each other and did not change over the first hour after infusion

RCV values were determined for each of the four biotin densities (6, 18, 54, and 162 μ g of biotinylation reagent per mL of autologous RBCs) at each of three time points for all subjects

Subject Number	Body Mass, (kg)	Height (cm)	Biotinylation Density, (μ g/mL RBC)	RCV 10 minutes	RCV 20 minutes	RCV 60 minutes
1	57.3	163.5	6	1,513	1,545	1,534
			18	1,508	1,535	1,539
			54	1,520	1,570	1,528
2	115.2	168.5	162	1,550	1,511	1,566
			6	2,216	2,140	2,194
			18	2,079	2,103	2,090
3	84.3	181.5	54	2,168	2,129	2,173
			162	2,107	2,149	2,202
			6	1,846	1,884	2,105
4	57.2	164.7	18	1,892	1,945	2,127
			54	1,903	2,005	2,162
			162	1,950	1,969	2,129
5	77.1	175.8	6	1,423	1,438	1,434
			18	1,397	1,414	1,438
			54	1,371	1,390	1,385
6	90.5	179.5	162	1,557	1,588	1,598
			6	1,565	1,580	1,588
			18	1,617	1,588	1,615
6	90.5	179.5	162	1,622	1,612	1,658
			6	2,105	2,173	2,106
			18	2,184	2,148	2,156
6	90.5	179.5	54	2,191	2,200	2,188
			162	2,101	2,172	2,162

Subject Number	Body Mass, (kg)	Height (cm)	Biotinylation Density, (µg/mL RBC)	RCV 10 minutes	RCV 20 minutes	RCV 60 minutes
7	100.92	184.6	6	2,520	2,577	2,258
			18	2,573	2,566	2,284
			54	2,437	2,565	2,330
			162	2,485	2,502	2,280
8	69.6	163.6	6	1,546	1,623	1,587
			18	1,539	1,565	1,541
			54	1,584	1,583	1,571
			162	1,631	1,630	1,647

Table 2
Mean RCV ratios to biotinylation density 6 µg/mL were not different among biotin densities 18 µg/mL, 54 µg/mL, and 162 µg/mL

Biotinylation density	Time (minutes)	Mean ratio	95 % CI of the mean ratio	
18 µg/mL	10	1.000	0.983	1.018
	20	0.992	0.974	1.009
	60	0.996	0.979	1.014
	Mean	0.996	0.978	1.015
54 µg/mL	10	1.006	0.985	1.028
	20	1.003	0.982	1.025
	60	1.006	0.985	1.028
	Mean	1.005	0.982	1.028
162 µg/mL	10	1.013	0.990	1.036
	20	1.000	0.977	1.023
	60	1.019	0.996	1.043
	Mean	1.011	0.986	1.036
Mean for all densities	10	1.006	0.985	1.028
	20	0.998	0.977	1.020
	60	1.007	0.986	1.028
	Mean	1.004	0.990	1.018

The mean RCV ratios did not differ significantly among the three time points ($p=0.50$) or among the three densities ($p=0.21$).