# Tracking donor RBC survival in premature infants: agreement of multiple populations of biotin-labeled RBCs with Kidd antigen mismatched RBCs

**John A. Widness**[1], **Demet Nalbant**[1], **Nell I. Matthews**[3], **Ronald G. Strauss**[1,2], **Robert L. Schmidt**[1], **Gretchen A. Cress**[1], **M. Bridget Zimmerman**[4], and **Donald M. Mock**[3]

[1]Department of Pediatrics, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA

[2]Department of Pathology, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA

[3]Department of Biochemistry & Molecular Biology and the Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR

[4]Department of Biostatistics, University of Iowa College of Public Health, Iowa City, IA

## Abstract

**Background**—Anemia, a common condition among critically ill premature infants, is affected by red blood cell (RBC) survival (RCS). We hypothesized that transfused allogeneic Kidd antigen mismatched RBCs would demonstrate the same concurrent RCS tracking as RBCs multi-labeled at separate, discrete low densities with biotin (BioRBCs).

**Methods**—Allogeneic RBCs from adult donors were labeled at four biotin densities, mixed, and transfused into 17 anemic premature infants. Nine of the donors and neonates were Kidd antigen mismatched. Serial post-transfusion blood samples were assayed for up to eight weeks by flow cytometry to track the survival of the proportions of Kidd antigen mismatched and biotinylated RBCs.

**Results**—Using linear mixed modeling to compare results, RCS of the three lowest BioRBC densities was similar to RCS by Kidd antigen mismatch and to one another. RCS of RBCs labeled at the highest BioRBC density was shortened.

**Conclusions**—RCS of different populations of RBCs can be tracked concurrently and reliably using the three lowest BioRBC densities. Although comparable RCS results can be achieved using Kidd antigen mismatches, BioRBCs are preferred for investigating neonatal anemias because biotin labeling of both allogeneic and autologous RBCs is possible.

CORRESPONDING AUTHOR: John A. Widness, MD, University of Iowa, Roy J. and Lucille A. Carver College of Medicine, 200 Hawkins Drive, 8807 JPP, Iowa City, IA 52242, Office: (319) 356-8102, Fax: (319) 356-4685, john-widness@uiowa.edu.

# INTRODUCTION

Severe anemia is a common clinical problem among premature and other critically ill infants. An important determinant of anemia is the duration of red blood cell (RBC) survival in the circulation. RBC survival (RCS) is determined in "cohort" or "population" RBC labeling experiments (1). Cohort labeling is achieved by *in vivo* labeling of newly produced RBC — most commonly by brief exposure to isotopically identifiable iron or glycine — released from the bone marrow all at the same time, and therefore all of the same age (1–3).

In humans, population RBC labeling is performed *ex vivo* by labeling all RBCs of all ages, with the most common, best known RBC label being $^{51}$Cr. The same result can be achieved post-transfusion by exploiting intrinsic differences in donor and recipient RBCs. Two of the most common examples of this are donor-recipient differences in major (4) or minor RBC surface antigen expression (5) or in Hb species, e.g., HbF versus HbA when assayed by flow cytometry using intact cells (4), or by HPLC using protein hemolysates (6). RBC kinetics may then determined by serially assaying the relative proportions of donor and recipient RBCs (7). These intrinsic methods for determining RCS have the advantage of avoiding the two most common problems resulting in artifactual shortening RCS, i.e., elution of label from the RBC surface and RBC injury from the labeling procedure. Unfortunately, intrinsic methods have the obvious limitation that they can only be applied post-allogeneic transfusion, and therefore are unsuitable for RCS studies of autologous RBC.

Prior to the 1970's most methods for determining RBC survival (RCS) relied on radiolabeling of RBCs with $^{51}$Cr and $^{32}$P. At about this time the use of radioactivity in research became unacceptable for vulnerable study populations including fetuses, infants, children, and pregnant women (8). As a result, studies of the pathophysiology and treatment of anemia in infants and children had few other options for direct measurement of RBC survival (RCS) or RBC volume. Fortunately, over the past 20 years, measurement of RCS using RBCs labeled with biotin (BioRBCs) has been shown to be practical, reliable, accurate, and safe. The biotin method has the nearly unique capability of being able to concurrently measure RCS for multiple populations of either autologous or allogeneic RBCs. These features of the biotin labeling method offer important advantages in investigations of the physiology, pathophysiology, and therapy of anemia. These capabilities are particularly important in newborn infants where determination of RCS is confounded by laboratory phlebotomy loss, growth, and transfusion.

The present study in very low birth weight (VLBW) premature infants compares the tracking of RBCs biotinylated at multiple different biotin densities with RBC tracking based on Kidd antigen differences. Based on our prior study of RCS of autologous multi-density labeled RBCs in adults (9), we hypothesized that RBCs labeled with low densities of biotin would exhibit the same RCS as allogeneic transfused Kidd antigen mismatched RBCs when concurrently transfused.

## RESULTS

Seventeen premature VLBW infants (9 females) were studied. Gestational ages ranged between 26 and 30 wk, and birth weights ranged between 0.39 and 1.31 kg (Table 1). These included 12 singleton (70%) and five twin infants. Body weights at the time of study ranged between 0.37 and 2.21 kg; mean birth weight $z$-score adjusted for gender and singleton or twin status was $-1.24 \pm 1.41$ (10). Mean study age ($\pm$ SD) at the time of the allogeneic study transfusion was $17 \pm 14$ d, with a range of 1 to 45 d. Prior to study, 10 infants had received between 1 and 3 RBC transfusions. All infant study subjects underwent RBC biotinylation followed by post-transfusion RBC tracking. Nine of the 17 had concurrent tracking of donor-recipient Kidd antigen mismatches along with the BioRBCs. For the Kidd mismatches it was always the donor RBCs, whether positive or negative, that were followed for survival. The nine mismatches include 1 Jka+, 3 Jkb+ and 2 Jkb− donors, and 3 double mismatches where donor was Jka−/Jkb+ and baby was Jka+/Jkb−.

### Tracking of post-transfusion RBC survival

Tracking survival of donor RBCs was performed separately for two groups of infants, each with a different reference study group. The first analysis included the subgroup of nine infants who had concurrent determinations of Kidd antigens and the four BioRBCs densities (Figure 1a). In this analysis, the Kidd antigen mismatches served as the *post hoc* analysis reference group (Table 2). With the exception of density 54 μg/ml at 2 wk, RCS tracking by the three lowest BioRBC densities agreed well and were not statistically different from the RCS tracking by Kidd antigen mismatches for the entire 6-wk study period. In contrast, the highest BioRBC density (162 μg/ml) was significantly more rapidly eliminated than Kidd antigen mismatched RBCs starting at the second week.

The second RBC survival tracking analysis performed included only the four BioRBC densities followed in all 17 infant study subjects, with the lowest BioRBC density (6 μg/ml) serving as the *post hoc* analysis reference group during the for 8-wk study period (Figure 1b). Selection of the 6 μg/ml BioRBC density was based on our prior autologous RCS data in adults supporting the current study's hypothesis that the lowest BioRBC densities accurately track true RBC survival (11). For 18 μg/ml BioRBC density, significant *post hoc* differences with the 6 μg/ml BioRBC reference group were detected at weeks 4, 6, 7, and 8 but not at week 5 (Table 3); For 54 μg/ml, statistically significant *post hoc* differences were observed at week 2 and thereafter except for at week 5. In comparison to the first analysis of the subgroup of nine infants with concurrent Kidd antigen mismatches, the greater number of subjects in the BioRBC analysis (n=17) generated much tighter confidence intervals (Tables 2 and 3), and therefore greater sensitivity in detecting *post hoc* statistically significant differences in the ratio of the 6 μg/ml reference group relative to the three other BioRBC densities differences. However, the differences are so small that they are unlikely to limit the utility of these densities for investigating RCS. For 18 μg/ml, differences were <10% and for 54 μg/ml differences were <20%. In contrast, for the 162 μg/ml the differences were >10% by week 2 and were >40% by week 7. These observations provide evidence of a substantial shortening of RCS, possibly due to RBC damage by biotinylation;

such an artifact will potentially lead to misleading conclusions when used to compare survival in different RBC populations.

To determine if there were differences in the nine infants included in the first group compared to the eight additional infants included in the second group, a subgroup analysis was performed. Comparisons performed included birth weight, gestational age at birth, postnatal age, gender, and singleton birth. No significant differences were detected (Table 1).

### Tracking of Kidd antigen double mismatches and HbF changes in selected infants

Four infants had unique RCS tracking data following their initial RBC transfusion (Figure 2). For two infants there was a donor-recipient Kidd antigen double mismatch (i.e., donor RBCs typed as Jka$^-$ Jkb$^+$ and recipient RBCs typed as Jka$^+$ Jkb$^-$) such that donor RBCs could be tracked by both Jka− and Jkb+ Kidd antigens. Both infants demonstrated concordance in the tracking using both Kidd antigens prior to their second RBC transfusion, 35 and 10 d after their first transfusion (Figure 2a and b). The other two infants had serial HbF$^-$RBC measurements tracked following their first transfusion. In both infants there was agreement between tracking of HbF$^-$RBC and the three lowest BioRBC densities (Figure 2c and d). Once again the heaviest BioRBC density declined more rapidly.

## DISCUSSION

Results of the present study support our hypothesis that RBCs biotinylated at low densities have normal post-transfusion survival compared to survival of non-biotinylated, Kidd mismatched RBCs. In contrast, the RBCs biotinylated at the heaviest density studied (162 μg/ml) have substantially shortened long-term RBC survival, possibly due to biotinylation injury. These conclusions are supported both by comparison to unlabeled RBCs detected by Kidd antigen mismatches and by relative comparison among the four densities of BioRBCs. This conclusion is also in agreement with our previous conclusion concerning shortened survival at higher biotinylation densities in normal adults when in hemodynamic and erythropoietic steady state; survival for the three lowest BioRBCs densities demonstrated close agreement with one another and the highest biotinylation density was substantially shortened (9). Although the present study demonstrates comparable minor RBC antigen RCS tracking results with lightly biotinylated RBCs, the versatility of biotin method in its ability to label both allogeneic and autologous RBCs and to concurrently track multiple RBC populations in all mammalian species are important advantages in investigations of the physiology, pathophysiology, and therapy of anemia (1).

The present RCS tracking study utilizing BioRBCs, Kidd antigen differences and HbF cell change over time extends our previous single BioRBC label infant study (12) by documenting for the first time in VLBW infants that multi-density BioRBCs can be reproducibly and predictably prepared from allogeneic RBCs and. Concurrent post-transfusion agreement of the tracking of the three lowest BioRBC densities with Kidd antigen mismatched RBCs indicates that biotinylation at low densities does not alter the survival of transfused RBC. Although there were minor differences among the three lowest BioRBC densities relative to the lowest BioRBC density as the reference (Figure 1b and

Table 3), the differences observed were small compared to the magnitude of reduced RCS usually deemed to be clinically unimportant. Accordingly, we contend that RBCs biotinylated at the three lowest biotin densities have comparable clinical utility such that they can be used interchangeably to concurrently and accurately track RCS of multiple RBC populations in infants.

The present findings have substantial implications for investigation of pathophysiology and treatment of neonatal anemia because acceptable methods for determining post-transfusion RBC kinetics in infants have been limited since 1970 (13). Between 1950 and 1970, RBC labeling with the radionuclide $^{51}$Cr became – and continues to be — the only FDA acceptable RBC labeling method for assessing RBC kinetics purposes, that is accepted for licensing by the FDA, e.g., licensing of modified RBC transfusion products or storage media (14, 15). Beginning about 1970, safety concerns regarding radiation exposure of vulnerable populations (fetuses, infants, children, and pregnant women) for research purposes led to the greatly restricted use and eventual abandonment of the use of radioactive RBC labeling in these populations. Consequently, although they commonly receive multiple RBC transfusions, critically ill VLBW infants have not been definitively studied using RBC kinetic methods. As demonstrated in the present study, the current generation of highly sensitive and accurate flow cytometers can detect, distinguish, and accurately enumerate different RBC populations using only a few microliters of blood and in only a few seconds of cytometry flow time. These features of flow cytometry offer the potential for resuming RBC kinetic studies in VLBW infants.

As demonstrated in the present study, biotin RBC labeling has the advantage of being able to measure the RCS of multiple RBC populations concurrently in the same individual. Potential applications of multiple RBC population labeling in infants includes being able to concurrently track RCS of the same allogeneic donor RBCs stored for different periods in multiply transfused study subjects. Similarly, biotin labeled autologous RBCs at one density can concurrently be used to track post-transfusion allogeneic donor RBCs labeled at a second biotin density. Although studies in adults have demonstrated the ability to concurrently measure radioactive $^{51}$Cr and nonradioactive $^{52}$Cr and/or $^{53}$Cr (16), these studies are technically challenging and expensive. As a result, multi-Cr-isotopic RBC labeling for concurrently determining RCS of more than one population of RBCs has rarely been applied.

Compared to $^{51}$Cr labeled RBCs, tracking of biotin-labeled RBCs can be accurately followed for longer periods of time. In normal healthy adults studied under steady-state erythropoiesis BioRBCs can be accurately quantitated up through the normal 120 d RBC life span (9). As demonstrated in the present study, this advantage also applies to other flow cytometry cell enumeration procedures in which there are intrinsic differences between donor and recipient RBCs, e.g., in RBC antigens or in Hb species (HbF and HbA). Because $^{51}$Cr undergoes both radioactive decay and variable RBC loss — ranging 0.5% to 2.5% of $^{51}$Cr per day as a result of elution and/or vesiculation of RBC membrane and intracellular hemoglobin (17) — RCS of $^{51}$Cr-labed RBCs requires correction for both these losses and as a result has only been empirically determined for 35 to 40 d.

From the standpoint of ease of use and feasibility, the biotin RBC labeling method has both advantages and disadvantages. Advantages include: 1) the biotinylating reagents required are inexpensive and commercially available; 2) the required measurement and synthesis equipment (a flow cytometer and sterile hood) are readily available; and 3) technician time required for analysis is modest (18, 19). Labeling RBCs with biotin, an essential B vitamin, has been shown to be nontoxic (11), and there have been no adverse clinical consequences other than asymptomatic, transient antibody formation in 15 to 20% of adults (20, 21). Disadvantages include: 1) the total human experience is limited and additional safety data are needed; and 2) human studies utilizing BioRBCs require submission of a Food and Drug Administration investigational new drug application.

Tracking of RCS as performed in this study does not permit the quantitative determination of classic RBC kinetic parameters such as mean potential lifespan or half-life ($T_{50}$). This is because studies in infants include confounding factors not present in adult steady state studies. These factors include ongoing significant laboratory blood loss (which was not measured in the present study) and hemodilution of labeled RBCs as a result of both RBC transfusions and growth-related blood volume expansion. When in future studies, RBC enrichment data such as those included in the present study are adjusted for accurate determinations of these confounders, pharmacodynamic modeling can be applied in deriving meaningful determinations of MPL and $T_{50}$. When these future RCS data are combined with serial Hb concentration measurements, the rates of RBC production and removal will further extend our understanding of the pathophysiological mechanisms of anemia (22).

In addition to not being able to derive meaningful MPL and $T_{50}$ determinations, the present study has several limitations. First, the study population consisted of a relatively small convenience sample of only 17 study subjects; Kidd antigen mismatch tracking was possible in only about half of these. Second, the 17 infants were a select group that may not be representative of all VLBW infants for all RBC transfusions these infants receive. With the exception of slowly evolving anemia for which all study subjects received non-emergent 15 ml/kg packed RBC transfusions, infants in the current study were clinically stable at time of and following transfusion. Third, discrepancies in changes in Kidd antigen differences and HbF RBC (but not BioRBC tracking) over time can only be accurately tracked until a second RBC transfusion is administered. Lastly, the study was limited to the pre-hospital discharge period, cutting short the time when BioRBC enrichment can be reliably measured, i.e., above 0.06% of total RBCs (9).

In summary, the present study documents that population labeling of donor RBCs with multiple low densities of biotin can be used to accurately track concurrent RCS, i.e., biotin densities of 6, 18 and 54 μg/ml. This conclusion is supported by results demonstrating close agreement in concurrently tracked non-labeled RBCs using Kidd antigen differences and multi-density labeled BioRBC populations. This conclusion is also supported by our previous observations in adults (9). The present study lays the groundwork for subsequent studies of survival of autologous RBCs, allogeneic RBCs and both simultaneously in neonates as well as other vulnerable populations including pregnant women, fetuses, infants, and children. RCS studies in the latter three groups are particularly challenging because confounding factors such as phlebotomy loss, growth, and transfusion commonly complicate

the determination of long-term RCS. Future studies of RBC survival in these never before studied, critically ill infants could shed light on new mechanisms contributing to neonatal anemia. Such studies are also likely to be important in developing a rational selection of RBC transfusion therapy (autologous versus allogeneic RBCs; fresh versus stored RBCs) and in evaluating the effectiveness of other therapies for neonatal anemia including delayed cord clamping, umbilical cord milking, erythropoietin treatment, and other future therapies.

## METHODS

The study was approved by the University of Iowa Committee on Research on Human Subjects (performance site) and by the Institutional Review Board at the University of Arkansas for Medical Sciences (analysis site). This included approval for administering Kidd mismatched RBCs. Informed written parental consent was obtained.

### Study subjects

A prospective, convenience sample of 122 eligible newborn infants included those <31 wk gestation for whom survival was anticipated and who were scheduled to receive a clinically indicated RBC transfusion. Infants who had received a prior RBC transfusion were not excluded. Infants with congenital anomalies were excluded.

### Study protocol

Following enrollment, a pre-transfusion discarded blood sample was analyzed to determine the Kidd antigen expression of study subjects. When a transfusion was expected, fresh (<7 d old) blood bank donor RBC units stored in AS3 preservative solution (Leukotrap RC System, Medsep, Covina, CA) were analyzed similarly for Kidd antigen expression. Kidd antigen mismatches were defined as differences in Kidd antigen expression between infant study subjects and adult donor blood. Blood bank RBC units identified as Kidd mismatched were maintained in the blood bank until a clinically indicated RBC transfusion was ordered. Occasionally this mismatch occurred by chance before the screening procedure could select a confirmed donor mismatch. Both qualitative and quantitative Kidd antigen testing was performed using the basic procedure described below.

Blood bank RBC units identified as Kidd mismatched were maintained in the blood bank until a clinically indicated RBC transfusion was ordered. Once ordered, the designated AS-3 donor unit underwent volume reduction by centrifugation to a packed red cell volume of ~0.85. Standard practice in our NICU is to transfuse with 15 ml/kg packed RBCs. In this study, the first 11 ml/kg of the transfusion was administered over 3 to 4 hr. The remaining allogeneic packed RBCs (minimum of 4 ml/kg, plus 2.8 ml to allow adequate volume for blood culture and hematological analysis) were brought to the research laboratory and four equal aliquots of approximately 1.7 ml/kg each were individually labeled at each of the four discrete biotin densities as previously described (9) and briefly summarized below. This BioRBC dose results in an enrichment of 2.0 to 2.5% for each BioRBC density.

Following the last wash step of biotinylation procedure described below, the supernatant fractions were removed and equal volumes of the four packed BioRBC populations, each with hematocrits of 75 to 85%, were combined, mixed, and then passed through an 18-

micron filter (Hemo-Nate, Utah Medical Products, Inc., Midvale, UT) to remove microaggregates. An aliquot of the labeled RBC mixture was cultured aerobically for bacteria (BD Bactec Plus Aerobic/F, Becton, Dickinson and Company, Sparks, MD). Within a few minutes after completing the 11 ml/kg clinical transfusion, the final 4 ml/kg of BioRBCs were infused over 10 min to complete the 15 ml/kg transfusion.

Beginning 24 hr after the 4 ml/kg BioRBC transfusion and ending at hospital discharge, leftover anti-coagulated whole blood from clinically ordered laboratory testing was salvaged twice-weekly for quantitative analysis of the proportions of BioRBCs and Kidd antigen present as described below.

### Biotinylation of RBCs

The method of biotin labeling RBCs has been previously described (11). Briefly, RBCs were washed with a carbonate buffered dextrose-sodium-phosphate wash solution to remove plasma proteins and resuspended at a hematocrit of 25%. For this adjustment hematocrit, and for the BioRBC RBC count prior to transfusion, hematological analysis was performed using the Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan). Four equal volume aliquots of RBCs were then incubated with four 3-fold serial dilutions of the biotinylating reagent, sulfo-succinimido-biotin (sNHS-biotin, Pierce Chemical Co., Rockford, IL) yielding final concentrations of 6, 18, 54, and 162 μg of sNHS-biotin/ml RBCs. The 3-fold density differences in the four densities of BioRBCs were previously demonstrated to provide complete separation from unlabeled RBCs and from the other biotin labeled RBC populations. Separation of the four BioRBC peaks enhances accuracy in the quantitation of the populations by avoiding need to correct for peak overlap. Maintaining the separate individual peaks as close together as possible maximizes the number of useful RBC populations.

### Quantitative flow cytometric RBC analysis

BioRBC. For each whole blood sample, the proportion of each population of biotinylated RBCs was determined by flow cytometric enumeration as previously described (9, 23, 24). Briefly, labeled RBCs were enumerated by flow cytometry using the FACSCalibur (BD Biosciences, San Jose, CA). Enrichment proportions were calculated as the ratio of the number of cells in each of the four manually specified population interval markers relative to the total number of RBCs counted. To enhance accuracy, $1 \times 10^6$ total events (i.e., cells) were counted. Under these conditions, the lower limit of quantitation for each BioRBC population is 0.06% of total RBCs (9).

Kidd RBC antigen mismatch. Kidd RBC antigens were selected for study because: 1) Kidd RBC antigens are reliably separated into distinct flow cytometric peaks using commercially available labeled antibodies; and 2) differences in donor-recipient antigen expression are predicted to frequent occur because of the relatively large percentages of individuals who either express or lack these antigens (i.e., potential donor-recipient "mismatches" are frequent). With respect to the latter, 47% of whites and 91% of blacks express the JKa antigen, with 23% and 9%, respectively, lacking the antigen. For JKb, 72% of whites and 43% of blacks express the antigen with 28% and 57%, respectively, lacking the antigen.

For flow cytometric analysis of both Jka[+] and Jkb[+] RBC, triplicate aliquots of 5 μl of the pre- and post-transfusion samples were washed to remove plasma proteins following a similar protocol as for BioRBCs (9, 24). RBCs were suspended in 0.3 ml of wash buffer with 2% BSA and incubated at room temperature overnight along with 5 μl of anti-Jka or anti-Jkb primary antibody (Immucor Inc., Norcross, GA). Overnight incubation was done with continuous mixing on a rotating wheel (Roller Drum Model TC-7, New Brunswick Scientific, Edison, NJ). Excess antibody was removed in two washes before subsequent incubation with a Alexa Fluor 488 conjugated anti-human secondary antibody (H10120, Invitrogen, Carlsbad, CA) at 37°C for 1 h. Prior to flow cytometry analysis, RBCs were washed three times and resuspended in wash buffer plus 2% BSA that had been pre-filtered through a 0.2 micron filter to final volume of 0.5 ml. The relative percentages of Jka or Jkb antigen positive and negative RBCs included under the two manually specified peaks relative to the total number of RBCs were determined from 200,000 gated events. Depending on the subject's Kidd mismatch status, sometimes the antigen negative peak was followed.

HbF[+]RBCs. Following allogeneic adult donor RBC transfusion, infant RBCs (that nearly exclusively contain HbF and are denoted as HbF[+]RBCs) are easily distinguished by flow cytometry from allogeneic adult donor RBCs (that nearly exclusively contain HbA). The percent HbF[+]RBCs in blood samples was determined by the Invitrogen Fetal Hemoglobin Test kit (Invitrogen Corporation, Camarillo, CA) using FITC-conjugated monoclonal antibody directed against HbF (25). These kits are intended to detect small percentages of HbF cells in maternal blood. In first demonstrating a linear response from 0 to 100% HbF cells, we were assured that these kits were reliable in the current study. The manufacturer's procedure was followed except for the following minor modifications. Duplicate 1.5 μl whole blood samples (~$5 \times 10^6$ cells) were fixed in 0.25 ml of 0.05% glutaraldehyde. RBCs were then permeabilized in 0.2 ml of 0.1% Triton X-100. Washed and pelleted RBCs were resuspended in 80 μl of PBS/BSA and incubated with HbF monoclonal antibody for 30 min at room temperature. RBCs were washed and resuspended in 0.4 ml of 2% paraformaldehyde prior to flow cytometric analysis. HbF[+]RBCs were enumerated with at least 100,000 events by a flow cytometry.

### Data handling and statistical analysis

Tracking of RCS survival was performed using linear mixed model analysis. The data used for the RCS tracking of the four different BioRBC populations and the two Kidd antigen mismatched RBC populations were expressed as a ratio relative to the sample at about 24 h after the 4 ml/kg transfusion, as recommended by ICSH for RCS measurement (17). Because blood samples for RCS tracking of individual infants were not obtained at precisely the same time (see description of leftover blood availability above), the ratios at the individual time points were grouped into the following intervals: 1 d to <3 d, 3 d to <7 d, and weekly intervals thereafter (e.g., 7 d to <14 d through 49 d to <56 d). Multiple data points for a subject within a given interval were treated statistically as replicates. Because the ratios did not have a normal distribution, a natural log transformation was applied to the data prior to linear mixed model analysis.

Two mixed models were fitted. One compared RCS tracking of Kidd antigen (Jka and Jkb) with the RCS tracking of the four BioRBC densities (6, 18, 54 or 162 μg/ml). The other compared RCS tracking of the lowest BioRBC density versus the three heavier BioRBC densities. The fixed effects in both models included density (or method), time, and density*time interaction. In both, the test for density*time interaction corresponds to testing whether RCS tracking over time shows non-parallel profiles among the densities and Kidd antigen. In addition to estimating the fixed effects in the mixed model, this analytic method permits selection of the covariance structure that best fits the relationship of the RCS data (i.e., BioRBC densities and Kidd antigen at different times) measured in the same subject. From the fitted models, tests of mean contrasts were performed to compare each of the BioRBC densities with Kidd antigen as the reference (first model) or the three heavier BioRBC densities versus reference density 6 μg/ml (second model) at each time interval. Because the tracking of RBC survival for Kidd antigen mismatches in the first model is valid only prior to the times before a second RBC transfusion was administered, time intervals for data included in this analysis extend only through post-transfusion week six while the second model was extended through post-transfusion week eight. The *p*-values for these tests were adjusted using the Bonferroni *post hoc* method to account for the number of comparisons. The means of the natural log transformed data were back-transformed to obtain the mean ratios for each biotin density and for the Kidd antigen at each time interval.

Descriptive summary results are presented as the mean ± standard deviation. A *p*-value <0.05 was considered statistically significant.

## Acknowledgments

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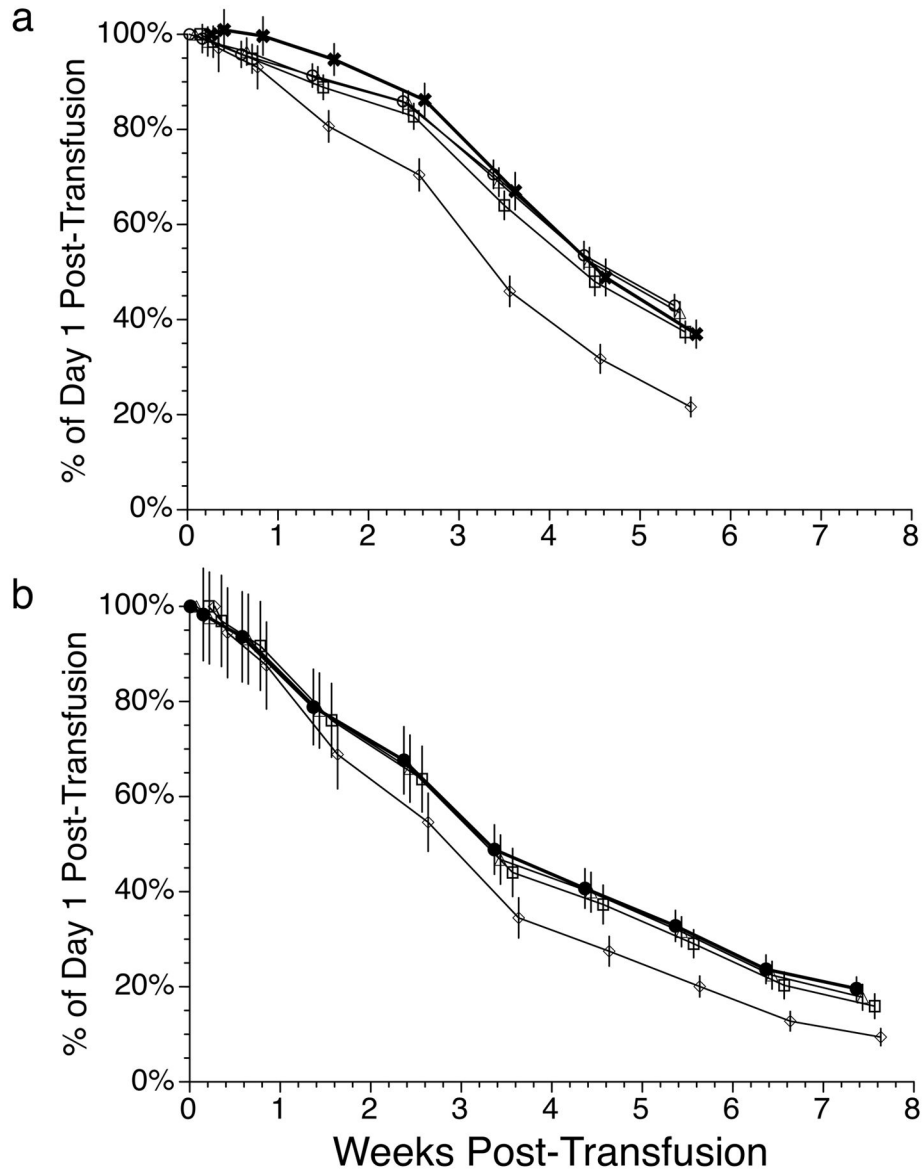
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**Figure 1.**
Comparison of mean (± SEM) RBC survival tracking using: a) Kidd antigen donor-recipient
mismatches as the reference control versus the four different BioRBC densities (n=9); and b)
the lowest BioRBC density as the reference control versus the three higher BioRBC
densities (n=17). The time of study for Panel a) is shorter because only data prior to the
second RBC transfusion is included. **x**, Kidd Antigen (Ref); open circles, BioRBC 6 μg/ml;
open triangles, BioRBC 18 μg/ml; open squares, BioRBC 54 μg/ml; open diamonds,
BioRBC 162 μg/ml.
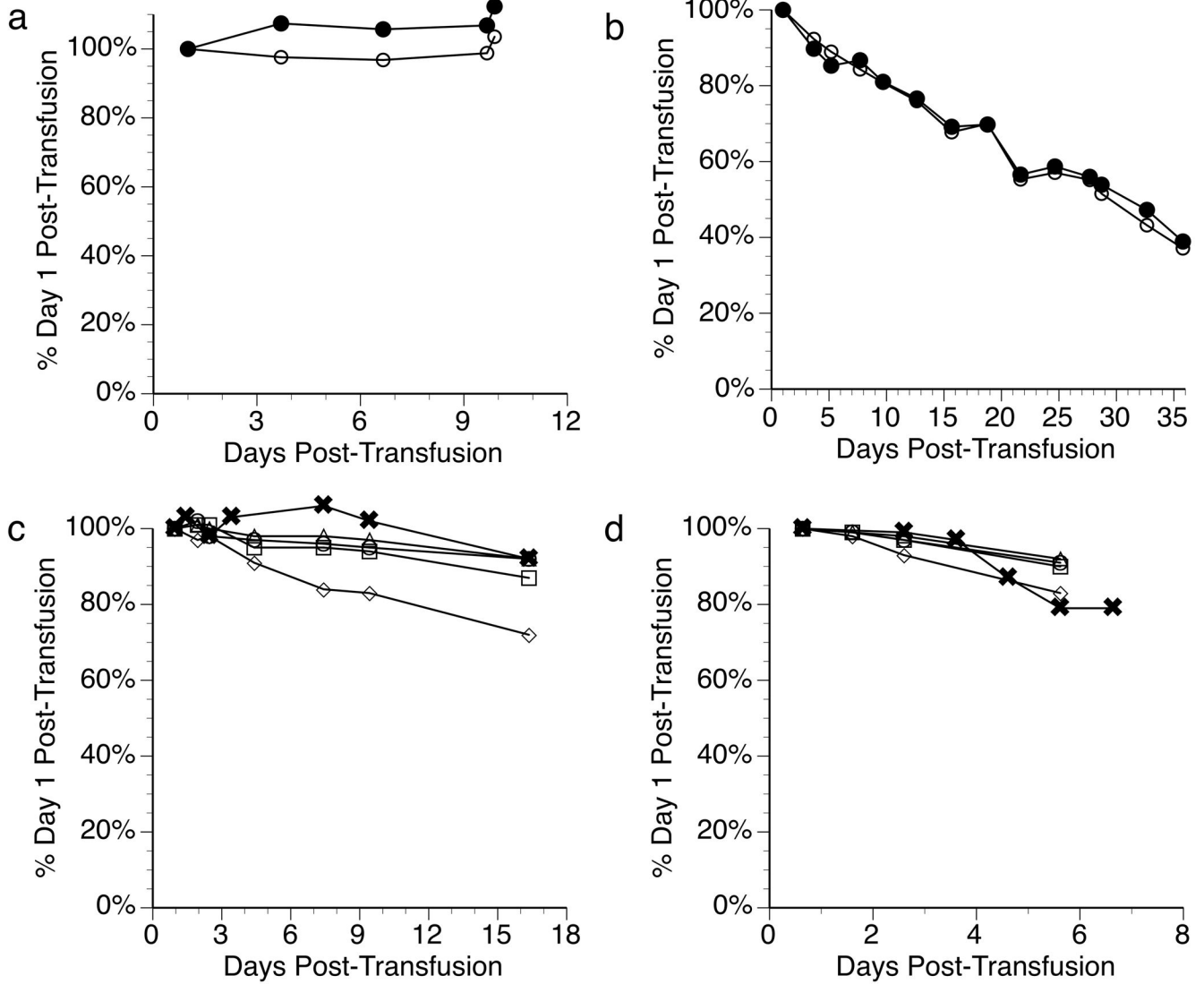
**Figure 2.**
Agreement of HbF and Kidd antigen RBC tracking for selected individual infants following their first RBC transfusion: a) Infant 11 (see Table 1) HbF negative cells versus the 4 BioRBC densities; b) Infant 7 HbF negative cells versus the 4 BioRBC densities; c) Infant 15 Kidd Jka cells versus Kidd Jkb cells; and d) Infant 4 Kidd Jka cells versus Kidd Jkb cells. (a and b) Open circle, Kidd Jka; closed circle, Kidd Jkb; (c and 2d) **x**, HbF Neg RBCs; open circles, BioRBC 6 µg/ml; open triangles, BioRBC 18 µg/ml; open squares, BioRBC 54 µg/ml; open diamonds, BioRBC 162 µg/ml.

**Table 1**

Premature VLBW infant subject and transfusion blood information.

| Subject number | Weight at Birth (kg) | Post-Menstrual Age at Birth (wk) | Post-Natal Age at Study (d) | Post-Natal Weight at Study (kg) |
|---|---|---|---|---|
| *Subjects with Kidd antigen mismatches at first transfusion* | | | | |
| 1 | 0.83 | 27.86 | 22 | 1.25 |
| 2 | 0.93 | 28.00 | 1 | 0.93 |
| 3 | 0.93 | 27.00 | 13 | 1.06 |
| 4 | 0.95 | 26.29 | 5 | 0.92 |
| 5 | 0.95 | 27.43 | 13 | 0.98 |
| 6 | 1.00 | 27.00 | 9 | 0.87 |
| 7[a] | 1.03 | 27.43 | 11 | 1.12 |
| 8 | 1.37 | 28.71 | 10 | 1.32 |
| 9 | 1.40 | 30.14 | 33 | 1.96 |
| Mean | 1.04 | 27.76 | 13.0 | 1.16 |
| SD | 0.20 | 1.13 | 9.5 | 0.34 |
| *Subjects without Kidd antigen mismatches at first transfusion* | | | | |
| 10 | 0.39 | 26.29 | 1 | 0.37 |
| 11[a] | 0.73 | 29.86 | 3 | 0.76 |
| 12 | 0.83 | 27.43 | 15 | 0.98 |
| 13 | 0.86 | 26.71 | 33 | 1.55 |
| 14 | 0.88 | 28.00 | 23 | 1.10 |
| 15 | 0.91 | 27.43 | 45 | 1.76 |
| 16 | 1.08 | 27.43 | 15 | 1.20 |
| 17 | 1.09 | 28.00 | 8 | 0.96 |
| Mean | 0.85 | 27.64 | 17.9 | 1.09 |
| SD | 0.22 | 1.07 | 15.2 | 0.44 |
| *All subjects* | | | | |
| Mean | 0.95 | 27.71 | 15.3 | 1.12 |
| SD | 0.23 | 1.07 | 12.3 | 0.38 |

[a]Indicates infants studied with HbF RBC tracking

**Table 2**

Comparison of concurrent post-transfusion RBC survival of Jka and Jkb RBC antigen donor-recipient mismatches with the four BioRBC densities.

| Time Post-Transfusion | Overall ANOVA *p*-value | Comparison BioRBC Density | Ratio Compared to Minor Antigen (CI) | *Post Hoc* *p*-value[a] |
|---|---|---|---|---|
| 1 to <3 d (n=8) | >0.99 | 6 μg/ml | 0.98 (0.90, 1.07) | >0.99 |
| | | 18 μg/ml | 0.98 (0.90, 1.06) | >0.99 |
| | | 54 μg/ml | 0.98 (0.90, 1.05) | >0.99 |
| | | 162 μg/ml | 0.96 (0.88, 1.06) | >0.99 |
| 3 to <7 d and (n=9) | 0.365 | 6 μg/ml | 0.96 (0.89, 1.04) | >0.99 |
| | | 18 μg/ml | 0.97 (0.89, 1.05) | >0.99 |
| | | 54 μg/ml | 0.95 (0.88, 1.02) | 0.701 |
| | | 162 μg/ml | 0.93 (0.85, 1.02) | 0.145 |
| Week 2 (n=8) | <0.0001 | 6 μg/ml | 0.96 (0.90, 1.03) | >0.99 |
| | | 18 μg/ml | 0.96 (0.90, 1.02) | 0.596 |
| | | 54 μg/ml | 0.94 (0.88, 0.99) | 0.034 |
| | | 162 μg/ml | 0.85 (0.79, 0.91) | <0.0001 |
| Week 3 (n=7) | <0.0001 | 6 μg/ml | 1.00 (0.92, 1.08) | >0.99 |
| | | 18 μg/ml | 0.99 (0.92, 1.07) | >0.99 |
| | | 54 μg/ml | 0.96 (0.89, 1.03) | >0.99 |
| | | 162 μg/ml | 0.82 (0.75, 0.89) | <0.0001 |
| Week 4 (n=3) | <0.0001 | 6 μg/ml | 1.05 (0.94, 1.18) | >0.99 |
| | | 18 μg/ml | 1.03 (0.92, 1.14) | >0.99 |
| | | 54 μg/ml | 0.96 (0.86, 1.06) | >0.99 |
| | | 162 μg/ml | 0.69 (0.61, 0.77) | <0.0001 |
| Week 5 (n=2) | <0.0001 | 6 μg/ml | 1.10 (0.93, 1.30) | >0.99 |
| | | 18 μg/ml | 1.07 (0.91, 1.25) | >0.99 |
| | | 54 μg/ml | 0.98 (0.85, 1.14) | >0.99 |
| | | 162 μg/ml | 0.65 (0.54, 0.78) | <0.0001 |
| Week 6 (n=2) | <0.0001 | 6 μg/ml | 1.16 (0.99, 1.36) | 0.067 |
| | | 18 μg/ml | 1.12 (0.97, 1.30) | 0.405 |
| | | 54 μg/ml | 1.01 (0.88, 1.16) | >0.99 |
| | | 162 μg/ml | 0.59 (0.49, 0.69) | <0.0001 |

[a]Using Bonferroni's *post hoc* method

**Table 3**

Comparison of concurrent post-transfusion RBC survival of lowest BioRBC density 6 μg/ml with the other three progressively greater BioRBC densities.

| Time Post-Transfusion | Overall ANOVA *p*-value | Comparison BioRBC Density | Ratio Compared to Minor Antigen (CI) | *Post Hoc* *p*-value[a] |
|---|---|---|---|---|
| 1 to <3 d (n=16) | 0.172 | 18 μg/ml | 0.99 (0.98, 1.00) | 0.689 |
| | | 54 μg/ml | 0.99 (0.97, 1.00) | 0.244 |
| | | 162 μg/ml | 0.96 (0.92, 1.01) | 0.281 |
| 3 to <7 d (n=17) | 0.003 | 18 μg/ml | 0.99 (0.98, 1.01) | >0.99 |
| | | 54 μg/ml | 0.98 (0.96, 1.00) | 0.085 |
| | | 162 μg/ml | 0.94 (0.89, 0.98) | 0.001 |
| Week 2 (n=17) | <0.0001 | 18 μg/ml | 0.99 (0.98, 1.01) | 0.890 |
| | | 54 μg/ml | 0.96 (0.94, 0.99) | 0.0002 |
| | | 162 μg/ml | 0.87 (0.83, 0.91) | <0.0001 |
| Week 3 (n=17) | <0.0001 | 18 μg/ml | 0.97 (0.94, 1.00) | 0.128 |
| | | 54 μg/ml | 0.94 (0.90, 0.98) | 0.0005 |
| | | 162 μg/ml | 0.81 (0.76, 0.90) | <0.0001 |
| Week 4 (n=17) | <0.0001 | 18 μg/ml | 0.96 (0.92, 0.997) | 0.013 |
| | | 54 μg/ml | 0.90 (0.84, 0.96) | 0.0002 |
| | | 162 μg/ml | 0.71 (0.63, 0.79) | <0.0001 |
| Week 5 (n=16) | <0.0001 | 18 μg/ml | 0.98 (0.94, 1.02) | >0.99 |
| | | 54 μg/ml | 0.92 (0.83, 1.01) | 0.189 |
| | | 162 μg/ml | 0.68 (0.58, 0.78) | <0.0001 |
| Week 6 (n=16) | <0.0001 | 18 μg/ml | 0.96 (0.94, 0.98) | <0.0001 |
| | | 54 μg/ml | 0.89 (0.84, 0.93) | <0.0001 |
| | | 162 μg/ml | 0.61 (0.55, 0.68) | <0.0001 |
| Week 7 (n=13) | <0.0001 | 18 μg/ml | 0.95 (0.91, 0.99) | 0.018 |
| | | 54 μg/ml | 0.86 (0.80, 0.92) | <0.0001 |
| | | 162 μg/ml | 0.54 (0.46, 0.64) | <0.0001 |
| Week 8 (n=11) | <0.0001 | 18 μg/ml | 0.91 (0.79, 1.04) | 0.506 |
| | | 54 μg/ml | 0.81 (0.68, 0.97) | 0.024 |
| | | 162 μg/ml | 0.48 (0.35, 0.65) | <0.0001 |

[a]Using Bonferroni's *post hoc* method