Erythropoietic Response to Endogenous Erythropoietin in Premature Very Low Birth Weight Infants^[S]

Kevin J. Freise, John A. Widness, and Peter Veng-Pedersen

Division of Pharmaceutics, College of Pharmacy (K.J.F., P.V.-P.) and Department of Pediatrics, College of Medicine (J.A.W.), The University of Iowa, Iowa City, Iowa

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ABSTRACT

Despite the common occurrence of anemia in very low birth weight (VLBW) infants, the erythropoiesis and Hb production rates and their relationship to plasma erythropoietin (EPO) concentrations remain unknown in these subjects. To determine these quantities, all blood removed by phlebotomy and administered by red blood cell (RBC) transfusion over the first 30 days of life was recorded in 14 ventilated VLBW infants born at 24 to 28 weeks of gestation. Discarded blood from frequent clinically ordered laboratory blood samples was used to construct plasma EPO, Hb, and RBC concentration-time profiles for each infant. A pharmacodynamic Hb mass balance model that accounted for the dynamic hematological conditions experienced by these infants was simultaneously fitted to the plasma EPO, Hb, and RBC concentrations from each individual subject,

while accounting for subject growth. Based on the model estimates, an average of 4.69 g of Hb was produced over the first 30 days of life, compared with 5.97 g removed by phlebotomies and 12.3 g administered by transfusions. These high transfusion amounts were consistent with a relatively short RBC life span and rapidly expanding blood volume with infant growth. The estimated mean body weight-scaled Hb production rate dropped nearly 3-fold after birth to 0.144 g/day·(kg)^{3/4}. Although only estimated in a subset of the subjects, the mean plasma EPO EC₅₀ of 28.5 mU/ml and maximal Hb production rate ($E_{\max}$) indicated that a severalfold increase in Hb production rate could be achieved with only a modest increase in plasma EPO concentrations.

The anemia of prematurity occurs in all very low birth weight (VLBW) premature infants (birth weight <1500 g) and is exacerbated by iatrogenic blood loss resulting from frequent laboratory blood sampling for managing clinical illness (Strauss, 1995; Lin et al., 2000; Madan et al., 2005; Widness et al., 2005). The resulting development of clinically significant anemia is managed by the administration of red blood cell (RBC) transfusions, creating dynamic changes in the Hb mass in these infants due to both the physical removal and administration of erythrocytes. In infants, as well as adults, RBCs are produced from erythroid progenitor cells located primarily in the bone marrow, although some residual erythropoiesis may still occur in the liver and spleen of preterm infants (Brugnara and Platt, 2003; Hoffman et al., 2005). The development and expansion of erythroid progen-

itor cells into mature RBCs is primarily controlled by erythropoietin (EPO), a 30.4-kDa glycoprotein hormone produced in response to oxygen need by the peritubular cells of the kidney in the adult and possibly also the liver in preterm infants (Brown, 1988; Moritz et al., 1997; Brugnara and Platt, 2003; Hoffman et al., 2005). In addition, under nondisease state conditions the mechanism of RBC death or removal from the circulation is primarily due to cellular senescence (i.e., age related cell death) (Landaw, 1988).

Despite the common occurrence of anemia in VLBW infants, the erythropoiesis rate in these subjects remains unknown. This is largely due to the complications in determining the erythropoiesis rate caused by frequent phlebotomies and RBC transfusions altering the RBC/Hb mass, and the effect of each phlebotomy on the RBC/Hb removal rate due to the life span-based disposition of RBCs (Landaw, 1988; Freise et al., 2007, 2008). Previous studies have demonstrated that on average 33.8 ml/kg blood is removed and 27.0 ml/kg blood is transfused during the first 4 weeks of life in infants born at a gestational age of less than 28 weeks (Madssen et al., 2000), and other studies have reported even higher phlebotomy blood loss volumes in the first 2 weeks of life

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ABBREVIATIONS: EPO, erythropoietin; rHuEPO, recombinant human erythropoietin; PD, pharmacodynamic; Hct, hematocrit; MSE%, mean percent standard error; AIC, Akaike's information criterion; VLBW, very low birth weight; RBC, red blood cell.

alone (Lin et al., 2000; Widness et al., 2005). Thus, the effects of the physical removal and administration of RBCs are substantial and cannot be ignored. Other complications in determining the erythropoiesis rate in preterm VLBW infants include increases in total blood volume as it expands with infant growth and the mixture of endogenously produced RBCs and exogenously administered adult donor RBCs after transfusion, the former of which generally has shorter life spans (Brugnara and Platt, 2003; Strauss et al., 2004).

Knowledge of the *in vivo* erythropoiesis and Hb production rates and their relationship to plasma EPO concentrations in these infants would provide an understanding of these subject's ability to compensate for phlebotomy blood loss, thereby providing a reference for evaluating the potential improvement of erythropoiesis through administration of recombinant human EPO (rHuEPO) and other erythropoiesis-stimulating agents. Because the administration of RBC transfusions carries infectious and noninfectious risks (Galel and Fontaine, 2006; Ohlsson and Aher, 2006), knowledge of the *in vivo* erythropoiesis rate is also important for assessing the potential therapeutic strategies to reduce or eliminate RBC transfusion risks. Thus, the objective of the current study was to estimate the *in vivo* erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations in preterm VLBW infants. To achieve this aim, the amount of Hb removed by phlebotomy and administered by RBC transfusion to 14 preterm VLBW infants was recorded and a pharmacodynamic (PD) Hb mass balance model was formulated that accounts for the dynamic hematological conditions experienced by these infants. The model was subsequently fitted simultaneously to each infant's observed endogenous plasma EPO, Hb, RBC count, and body weight profiles over time to estimate the erythropoiesis rate and its relationship to endogenous plasma EPO concentrations.

Materials and Methods

Subjects. A consecutive sample of 14 inborn infants between 24 and 28 weeks of gestation being cared for in the Neonatal Intensive Care Unit at the University of Iowa Children's Hospital (Iowa City, IA) were enrolled between February 2007 and February 2008. Additional inclusion requirements were treatment with expectation of survival and moderate-to-severe respiratory distress requiring ventilation. Infants were excluded who had hematological disease (except for anemia associated with phlebotomy blood loss and prematurity); alloimmune hemolytic anemia; diffuse intravascular coagulation; transfusion requirements that were emergent, which did not allow controlled sampling; or had received erythropoiesis-stimulating agents. The study was approved by the University of Iowa Human Subject Internal Review Board, and all procedures were carried out in accordance with the Declaration of Helsinki. All subjects' parents or legal guardians signed informed consent.

Study Procedures. Phlebotomy blood samples from study subjects were weighed (AE50 Mettler balance scale; Mettler-Toledo Inc., Columbus, OH) and recorded immediately after collection from birth through 30 to 37 days of life. The collection tube weights were subtracted from the samples and converted to a blood volume based on the specific gravity of blood of 1.05 g/ml (Trudnowski and Rico, 1974). If a phlebotomy blood sample was mistakenly not weighed (~4% of all samples), the mean sample weight for the type of clinical test drawn was substituted. An additional 38 μ l of blood was added to each phlebotomy collected by capillary heel stick to account for blood wiped away before collection and bleeding after collection. The

38 μ l of blood was determined by weighing the volume of blood estimated to be equivalent (as determined by visual assessment) to the amount routinely removed from the skin before collection and loss after sample collection by capillary heel stick. In addition, the same amount of blood was added to samples collected from indwelling arterial or venous lines due to loss by clearing of the catheter before collection. Analysis of catheter fluid used to clear the lines from a sample of infants indicated that from 10 to 114 μ l (median, 38 μ l) of blood was lost with each blood sample drawn from an indwelling catheter.

The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed by the Hb concentration measured in the blood sample drawn closest to the time of blood sampling. Concentrations of Hb and RBCs were measured from several sources, including clinically ordered blood gases and electrolytes (Hb only) by using a Radiometer ABL 625 blood gas analyzer (Radiometer America, Inc., Westlake, OH), clinically ordered and study protocol-driven complete blood counts by using an Advia 120 hematology system (Bayer Corp., Tarrytown, NY), and excess blood recovered from other clinical tests by using a Sysmex XE-2100 automatic hematology analyzer (Sysmex Corporation, Kobe, Japan) operated in normal [if sufficient blood volume (>200 μ l) was available] or capillary mode. If blood collected at the same time was measured by multiple instruments, the average Hb or RBC concentration from all instruments was used. We have demonstrated previously that Hb and RBC concentrations measured on recovered blood by using the Sysmex XE-2100 is stable for up to 72 h at 4°C or room temperature and unaffected by the use of EDTA or heparin as an anticoagulant or operation in capillary mode (Freise et al., 2009). In addition to the Hb and RBC concentrations from clinically ordered tests, research blood samples were collected weekly and before and approximately daily after the first RBC transfusion for 10 days to ensure adequate sampling density. However, if a complete blood count was ordered by the attending physician(s) on the corresponding days, then research blood samples were not collected to avoid duplicate sample collection. The total amount of blood removed by the research blood samples was limited to less than 1.6 ml/week/kg.

Plasma samples for EPO concentration determination were also collected by centrifugation from excess blood recovered from the clinical tests and research samples described above. If the plasma volume from the sample was insufficient to conduct a plasma EPO determination, then the plasma was pooled with other samples within an approximately 8-h time window. The "collection time" for these pooled plasma samples used for the data analysis was the weighted average (based on the relative plasma volume contribution to the total sample) of the collection times of the individual samples that made up the pooled samples. Plasma EPO concentrations were measured using a double antibody radioimmunoassay procedure as described previously (lower limit of quantification 1 mU/ml) (Widness et al., 1992).

The volumes of packed RBCs [85% hematocrit (Hct)] administered by syringe (30 ml or 60-ml BD syringe; BD Biosciences, Franklin Lakes, NJ) and infusion pump, as well as the start and stop times of all RBC transfusions were recorded. The Hb mass administered to individual infants was calculated based on the measured Hb concentrations of the transfusate, or if not directly measured, Hb mass was based on a typical Hb concentration of 28.3 g/dl. The decision to treat an individual subject's anemia by administration of RBC transfusions was made by the physician responsible for the subject's patient care according to Neonatal Intensive Care Unit guidelines (Strauss, 2008). From the known amounts of Hb removed by phlebotomy and administered by transfusion to each infant and the observed Hb, RBC, plasma EPO concentration, and body weight profiles over time, the erythropoiesis and Hb production rates and their relationships to endogenous plasma EPO concentrations were estimated using the PD Hb mass balance model described below.

Pharmacodynamic Hb Mass Balance Model. The PD Hb mass balance model assumed that the behavior or disposition of the Hb

and RBCs in the absence of phlebotomies was life span-based (i.e., based on removal of RBCs from the circulation through cellular aging/senescence) (Landaw, 1988; Krzyzanski et al., 2006; Freise et al., 2008). A time invariant “point distribution” (i.e., no variability) of RBC life spans was assumed (Krzyzanski et al., 2006; Freise et al., 2007). The Hb mass or amount in the infants was modeled as the summation of two separate components: 1) Hb contained in RBCs produced by the infant in vivo ($Hb_{in vivo}$) and 2) Hb contained in transfused RBCs (Hb_{trans}).

The Hb production rate of the first Hb component, $Hb_{in vivo}$, was assumed to be proportional to body weight or mass scaled to the 3/4 power, as many metabolic processes are in physiology and pharmacokinetics (Savage et al., 2004; Meibohm et al., 2005; Anderson and Holford, 2009), to account for the changing body mass of the fetus/infant as the subject matured. Before birth, the body weight-scaled Hb production rate was assumed to be constant and was denoted by $k_{prod}^{in utero}$. Although after birth the body mass-scaled Hb production rate, denoted by $f_{prod}^{ex utero}(t)$, was assumed to be a function of time (t) through changes in the plasma EPO concentration [$C_{EPO}(t)$]. Thus, the total body Hb production rate [$f_{prod}^{total}(t)$] is a function of time through changes in both the body mass and the plasma EPO concentration over time as given by

$$f_{prod}^{total}(t) = f_{prod}(t) \times m(t)^{3/4} \quad (1)$$

where $m(t)$ is the body mass and

$$f_{prod}(t) = \begin{cases} k_{prod}^{in utero} & \text{if } t \leq 0 \\ f_{prod}^{ex utero}(C_{EPO}(t)) & \text{if } t > 0 \end{cases} \quad (2)$$

and $t = 0$ denotes the time of birth. A Michaelis-Menten or hyperbolic E_{max} model of plasma EPO concentration changes over time was used for $f_{prod}^{ex utero}(t)$; however, due to the limited plasma EPO concentration range observed for many subjects, the model was parameterized as

$$f_{prod}^{ex utero}(t) = \frac{p_1 \times C_{EPO}(t)}{1 + p_2 \times C_{EPO}(t)} \quad (3)$$

such that $p_1 = E_{max}/EC_{50}$ and $p_2 = (EC_{50})^{-1}$, where E_{max} is the maximal body weight-scaled Hb production rate and EC_{50} is the plasma EPO concentration that results in 50% E_{max} . This parameterization allows for the nonlinear production rate function given by eq. 3 to reduce to a linear function by setting $p_2 = 0$ when only operating in the approximately linear range of the E_{max} model (i.e., when $C_{EPO} \ll EC_{50}$; see Data Analysis below).

Let the time between production of progenitor cells outside the systemic circulation to release of the subsequently produced RBC into the circulation be denoted by a and the RBC life span of cells produced in vivo be denoted by $L_{in vivo}$. Hence, the time between production of progenitor cells and age-related death of RBCs, denoted by b , is given by $b = a + L_{in vivo}$. Then, in the absence of any phlebotomies, the rate of change in the Hb amount that was produced in vivo is given by (Freise et al., 2007)

$$\frac{dHb_{in vivo}}{dt} = Hb'_{in vivo}(t) = f_{prod}^{total}(t - a) - f_{prod}^{total}(t - b) \quad (4)$$

with initial conditions given by

$$Hb_{in vivo}(0) = \int_{t-b}^{t-a} f_{prod}^{total}(u) du|_{t=0} = \int_{0-b}^{0-a} f_{prod}^{total}(u) du$$

Thus, the input rate [$f_{prod}^{total}(t - a)$] and the output rate [$f_{prod}^{total}(t - b)$] of the in vivo Hb amounts are simply time shifted total body Hb production rates.

The second Hb mass component, the Hb from the RBC transfusions (Hb_{trans}), was accounted for through superposition by adding the Hb mass transfused at each transfusion and then accounting for a linear rate of decline of the transfused cells. The linear rate of decline arises from assuming a constant Hb production rate and a

constant RBC life span in the RBC donor subjects. Thus, in the absence of any phlebotomies

$$Hb_{trans}(t) = \begin{cases} Hb_{trans}(t) & \text{for } t = T_{Ti} \\ Hb_{trans}(t) + F_T \times Hb_{Ti} & \text{for } t = T_{Ti} + \epsilon \end{cases} \quad i = 1 \text{ to } m \quad (5)$$

where T_{Ti} is the time of the i th transfusion, Hb_{Ti} is the amount Hb administered at the i th transfusion, ϵ denotes an infinitesimally small time increment, $0 \leq F_T \leq 1$ is the fraction of transfused RBCs surviving immediately beyond the transfusion (e.g., if a portion of the RBCs were damaged in storage and removed by the reticuloendothelial system shortly after transfusion), and m is the number of transfusions. Although all RBC transfusions were administered over a 3- to 4-h time period, the effect of the transfusion on the Hb mass was approximated assuming that the cells were administered as a bolus. The linear rate of decline of the amount transfused Hb is given by

$$Hb'_{trans}(t) = -F_T \times \sum_{i=1}^m Hb_{Ti} \times f_{trans}(t - T_{Ti}), \quad Hb_{trans}(0) = 0 \quad (6)$$

with

$$f_{trans}(t) = \begin{cases} \frac{1}{L_{trans}} & \text{for } 0 \leq t < L_{trans} \\ 0 & \text{otherwise} \end{cases} \quad (7)$$

where L_{trans} is the RBC life span of the transfused cells from the donor subject. The storage age of the donor blood was not accounted for in the model because long-term RBC survival is unaffected and the short-term recovery is still within AABB requirements (Luten et al., 2008; Roback et al., 2008). By superposition, summation of $Hb_{in vivo}(t)$ and $Hb_{trans}(t)$ gives the total amount of Hb present in the systemic circulation:

$$Hb_{total}(t) = Hb_{in vivo}(t) + Hb_{trans}(t) \quad (8)$$

The total number of RBCs present in the systemic circulation, $RBC_{total}(t)$, was given by dividing the $Hb_{in vivo}(t)$ and the $Hb_{trans}(t)$ by the corresponding mean corpuscular Hb, that is, $MCH_{in vivo}$ and MCH_{trans} , respectively, as given by

$$RBC_{total}(t) = Hb_{in vivo}(t)/MCH_{in vivo} + Hb_{trans}(t)/MCH_{trans} \quad (9)$$

A separate MCH was used for the infant in vivo and adult transfused RBCs because infant cells are generally larger and contain more Hb than adult cells (Hoffman et al., 2005).

The above-presented equations are only applicable in the absence of any phlebotomies. Because the studied infants were subjected to numerous and frequent phlebotomies for clinical testing purposes, corrections to the above-presented equations were needed. To do so, at the time of each phlebotomy $Hb_{in vivo}(t)$ was corrected as

$$Hb_{in vivo}(t) = \begin{cases} Hb_{in vivo}(t) & \text{for } t = T_{Pj} \\ Hb_{in vivo}(t) - Hb_{Pj} \times \frac{Hb_{in vivo}(T_{Pj})}{Hb_{total}(T_{Pj})} & \text{for } t = T_{Pj} + \epsilon \end{cases} \quad j = 1 \text{ to } n \quad (10)$$

and $Hb_{trans}(t)$ was corrected as

$$Hb_{trans}(t) = \begin{cases} Hb_{trans}(t) & \text{for } t = T_{Pj} \\ Hb_{trans}(t) - Hb_{Pj} \times \frac{Hb_{trans}(T_{Pj})}{Hb_{total}(T_{Pj})} & \text{for } t = T_{Pj} + \epsilon \end{cases} \quad j = 1 \text{ to } n \quad (11)$$

where T_{Pj} is the time of the j th phlebotomy, Hb_{Pj} is the amount of Hb removed by the j th phlebotomy, and n is the total number of phlebotomies.

In addition, the negative terms in the differential eqs. 4 and 6 presented above [i.e., $f_{prod}^{total}(t - b)$ and $Hb_{Ti} \times f_{trans}(T - T_i)$, respectively] must be corrected for Hb removed by the phlebotomies. These

negative terms represent the output of Hb from the system due to cell age-related death of RBCs and thus if not corrected, the Hb will be “removed” twice, once due to the correction presented above in eqs. 10 and 11 at the time of each phlebotomy and a second time when the Hb would have been removed from the systemic circulation in the absence of the phlebotomies due to age-related death of RBCs. Because the cells physically removed by the phlebotomy cannot be removed again through the differential equation terms that represent removal by cell death (Krzyzanski and Jusko, 2002), these terms must be modified so that the equations remain correct. Let F_{P_j} be the fraction of Hb remaining immediately after the j th phlebotomy relative to the amount present immediately before the j th phlebotomy, thus

$$F_{P_j} = \frac{\text{Hb}_{\text{total}}(T_{P_j}) - \text{Hb}_{P_j}}{\text{Hb}_{\text{total}}(T_{P_j})} \quad (12)$$

In addition, if F_{P_j} are ordered from the first to the last phlebotomy such that $T_{P_{j+1}} > T_{P_j}$, then the exact phlebotomy correction factors to multiply these negative terms with in the differential equation is given by

$$\text{Phlebotomy correction factor} = \begin{cases} \prod_{j=k}^l F_{P_j} & \text{if } l \geq k \text{ and } T_{P_k} < t \\ 1 & \text{otherwise} \end{cases} \quad (13)$$

where k is the first phlebotomy after entry of the cells of interest into the systemic circulation and l is the last phlebotomy before the current time t (see Appendix for derivation). Therefore, as long as $T_{P_k} < t$, k is the first phlebotomy after time $t - L_{\text{in vivo}}$ for the $f_{\text{prod}}^{\text{total}}(t - b)$ term and the first phlebotomy after time $t - T_i$ for each $\text{Hb}_{T_i} \times f_{\text{trans}}(t - T_i)$ term. The presented phlebotomy correction factor after $l - k + 1$ phlebotomies is consistent with formulas previously derived after only one or two phlebotomies (Freise et al., 2007, 2008).

Finally, the amounts estimated from $\text{Hb}_{\text{total}}(t)$ and $\text{RBC}_{\text{total}}(t)$ were converted into the observed concentrations by the model estimated total blood volume. The total blood volume, V_{total} , was assumed to be proportional to the infant body mass (Sisson et al., 1959; Meibohm et al., 2005), and was given by

$$V_{\text{total}}(t) = m(t) \times V_N \quad (14)$$

where V_N is the body weight or mass-normalized blood volume.

Data Analysis. All modeling and simulations were conducted using WINFUNFIT, a Windows (Microsoft, Redmond, WA) version evolved from the general nonlinear regression program FUNFIT (Veng-Pedersen, 1977), by using ordinary unweighted least-squares fit to each individual subject's Hb and RBC concentration-time profile. The amount of Hb removed and administered by each phlebotomy and transfusion, respectively, at the time of removal or administration was accounted for by WINFUNFIT by using a generalized events processing module. The events processing module integrates the differential equation exactly up to the time of the event before adding or removing the appropriate amount and then continuing on integrating the differential equation from the new initial conditions set immediately after each successive event.

The EPO plasma concentrations were nonparametrically represented using a generalized cross-validated cubic spline function

(Hutchinson and deHoog, 1985). The infant body weight after birth was represented by a fourth-order polynomial fit to the observed body weight data to interpolate between body weight observations and provide a smooth function of total blood volume. To account for the in utero body masses, which are needed to calculate $f_{\text{prod}}^{\text{total}}(t)$ when $t \leq 0$, a power function was fitted to the mean body weights of over 10,000 live singleton births 22 to 32 weeks of gestational age (Arbuckle et al., 1993). Then for each infant, the in utero body masses were calculated based on their gestational age and linearly scaled such that function predicted birth body mass was continuous with the body weight at birth. Thus, both plasma EPO concentrations and body weights acted as forcing functions in the model.

To account for the fact that 24-h post-transfusion recoveries are generally less than 100% (Hess et al., 2003), F_T was fixed to 0.875, the midpoint between 100% recovery and the AABB requirements that transfused RBCs must exhibit 75% or greater recovery after storage (Roback et al., 2008). The life spans of the adult transfused (L_{trans}) was fixed to 70.8 days, the midpoint of the estimated life spans of 56.4 and 85.2 days of transfused adult RBCs in preterm infants (Bard and Widness, 1997; Strauss et al., 2004). In addition, the time between production of progenitor cells outside the circulation to release of the subsequently produced RBCs into the circulation (a) was set equal to 3 days based on previous estimates (Izak, 1977; McKenzie, 1988; Hoffman et al., 2005; Krzyzanski et al., 2005). The $\text{MCH}_{\text{trans}}$ parameter was set equal to the measured MCH for each unit of transfused blood, or if not measured for a particular unit of blood then the mean value of all the measured units (27.5 pg/cell). For each subject, the decision to use a nonlinear (p_2 -estimated) or linear ($p_2 = 0$) body weight-scaled Hb production rate function (eq. 3) was made using the Akaike's information criterion (AIC). All remaining parameters of the model (i.e., $L_{\text{in vivo}}$, V_N , $\text{MCH}_{\text{in vivo}}$, $k_{\text{prod}}^{\text{in utero}}$, and $E_{\text{max}}/\text{EC}_{50}$) were estimated in all subjects.

To summarize the uncertainty in the individual subject parameter estimates, the mean percent standard error (MSE%) of the estimate was calculated for each parameter as

$$\text{MSE}\% = \frac{1}{n} \times \sum_{i=1}^n \frac{\text{SE}_i}{|P_i|} \times 100 \quad (15)$$

where SE_i and P_i are the standard error of the parameter and the estimate of the parameter for the i th subject, respectively, and n is the number of subjects for which the parameter was estimated.

Results

Subject Characteristics. The mean gestational age of the 14 infant study subjects was 27 weeks (range, 25.0–28.6) and mean birth weight was 0.840 kg (range, 0.548–1.49). Five males and nine females were studied. A summary of the number of phlebotomies and RBC transfusions and the corresponding amounts of Hb removed and administered per study subject, respectively, are displayed in Table 1. All subjects who received transfusions were administered more Hb than was removed by phlebotomy. Approximately twice as much Hb was administered by transfusion as removed by

TABLE 1
Individual subject Hb, phlebotomy, and transfusion characteristics during study period ($n = 14$)

	First Hb Conc. after Birth	Study Period	No. of Phlebotomies	No. of Transfusions	Total Amount of Hb Removed by Phlebotomies	Total Amount of Hb Transfused
	<i>g/dl</i>	<i>days</i>			<i>g</i>	
Mean	16.3	32.1	143	4.07	6.27	14.4
S.D.	2.3	2.46	56.1	2.50	2.58	6.77
Min.	11.6	29.7	48	0	2.12	0.00
Max	19.3	37.3	239	8	10.6	24.3

Fig. 1. Mean rate of phlebotomy blood loss versus subject age.

phlebotomy. In 54 of the 57 transfusions administered, the volume of packed RBCs (85% Hct) administered was 15 ml/kg. The mean storage age of all RBC transfusions was 11.0 days (range, 2–42). Due to the severity of cardiorespiratory disease encountered in the first few weeks of life, the majority of RBC transfusions administered to these infants occurred at an Hct of approximately 35%. During the entire approximately 1-month study period, the average number of phlebotomies performed per subject per day was 4.5 (range, 1.6–7.1). The mean rate of blood removal by phlebotomy began very high at 10.1 ml/day/kg body weight on the first day of life and subsequently decreased over the next few days (Fig. 1). On average, 48.3% of the blood removed in the first 30 days of life was removed during the first 7 days of life. The mean daily rate of phlebotomy blood removal over the study period was 1.72 ml/day/kg body weight. The plasma EPO concentrations were variable and declined approximately 3-fold from 45 mU/ml immediately after birth to an average of 15.5 ± 6.55 (mean $\pm$ S.D.) mU/ml from 5 days of age onward (Fig. 2).

Pharmacodynamic Hb Mass Balance Model. The Hb mass balance model fit to the Hb and RBC concentration-time profiles, along with the plasma EPO concentration and body weight data (with fitted curves) for two representative subjects are displayed in Fig. 3 (individual subject data fittings are given in supplemental material, Supplemental Figs. 1–14). General agreement between the model fit and the Hb and RBC concentrations was observed (R^2 range 0.868–0.978). The administrations of the RBC transfusions are indicated by the vertical lines in the model predicted Hb and RBC concentration-time profiles. The rapid decline in the Hb and RBC concentrations after each transfusion is due to a combination of phlebotomy blood loss, blood volume expansion with growth (minor component), and RBC age-related death of both endogenously produced and transfused RBCs. Individual subject's Hb concentration-time profiles contained on average 91 Hb (range, 28–172), 27 RBC (range, 13–44), 50 plasma EPO (range, 10–96) concentration and 32 body weight (range, 30–37) measurements. The observed plasma EPO concentrations and body weights were also well represented by the fitted cross-validated cubic spline and fourth order polynomial, respectively (Fig. 3). The estimates of the parameters are displayed in Table 2 (individual subject parameters are given in supplemental material, Supplemen-

tal Table 1). The parameters were well estimated with a MSE% of <15% for all but the Hb production rate function parameters. The relatively high MSE% for $E_{\max}/EC_{50}$ is due to a single subject having a high standard error of 1300%. Calculation of the MSE% for $E_{\max}/EC_{50}$ without this subject results in a MSE% of only 24.6%. The EC_{50}^{-1} parameter, and thus $E_{\max}$ and EC_{50} secondary parameters, were only determined in six subjects where the nonlinear Hb production rate function (eq. 3) was preferred over a linear function based on AIC. The estimated amounts of Hb present at birth and produced (and released into the circulation) over the first 30 days of life, as well as the observed amounts of Hb removed by phlebotomy and administered by RBC transfusion, are displayed in Table 3. By 30 days of age, the estimated cumulative amount of Hb released into the circulation in the 14 VLBW study subjects ranged from 0.64 to 14.2 g/kg birth weight.

Discussion

The in vivo erythropoiesis rate and its relationship to endogenous plasma EPO concentrations in VLBW infants over the first 30 days of life was successfully estimated using a PD-based Hb mass balance model with 1) detailed accounting of all blood removed and transfused; 2) frequently sampled Hb, RBC, and plasma EPO concentration-time profiles created through recovery of excess blood collected; and 3) recording of serial body weights to account for growth and blood volume expansion. The estimated mean postbirth body weight-scaled Hb production rate over the first 30 days of life (average $f_{\text{prod}}^{\text{ex utero}}$) of $0.144 \text{ g/day} \cdot \text{kg}^{3/4}$ of body weight can serve as a quantitative reference for the erythropoietic ability of VLBW infants, under conditions of mild anemia due to receiving clinically ordered RBC transfusions, to compensate for Hb removed by phlebotomy and Hb dilution due to blood volume expansion as a result of growth. Furthermore, this reference and the postbirth Hb production rate parameters estimates ($E_{\max}/EC_{50}$ and EC_{50}^{-1}) are both important for assessing the potential to reduce or eliminate RBC transfusion risks through administration of exogenous rHuEPO or other erythropoiesis-stimulating agents.

In healthy term infants, the Hb production rates have been shown to decrease approximately 8-fold at day 10 of life relative to day 1 of life (Garby et al., 1963). A decrease was

Fig. 2. Plasma EPO concentrations versus subject age. The solid line represents a nonparametric smoothing spline fit to the observed data (circles).

Fig. 3. Pharmacodynamic Hb mass balance model fit to representative subjects. The symbols represent the observed data and the lines represent the model fit. A (left) and B (right) are different subjects.

TABLE 2
Estimated parameter summary from the pharmacodynamic Hb mass balance model ($n = 14$)

	$L_{in vivo}$	V_N	$MCH_{in vivo}$	$k_{prod}^{in utero}$	E_{max}/EC_{50}	$1/EC_{50}^a$	$Avg. \cdot f_{prod}^{ex utero b}$	$E_{max}^{a,b}$	$EC_{50}^{a,b}$
	days	ml/kg	pg/cell	g/day · kg ^{3/4}	g · ml/day · mU · kg ^{3/4}	ml/mU	g/day · kg ^{3/4}	g/day · kg ^{3/4}	mU/ml
Mean	65.8	97.7	37.5	0.414	0.0140	0.062	0.144	0.566	28.5
S.D.	42.7	31.5	3.1	0.156	0.0127	0.037	0.109	0.372	28.8
MSE%	11.6	6.2	9.2	7.4	123	33.6	N.A.	N.A.	N.A.

N.A., not applicable.
^a Only determined in 6 of 14 subjects based on AIC.
^b Secondary parameter.

TABLE 3
Observed and estimated Hb amounts over the first 30 days of life from the pharmacodynamic Hb mass balance model ($n = 14$)

	Estimated Amount Present at Birth	Estimated Amount Produced and Released into the Circulation	Observed Amount Removed	Observed Amount Transfused
	g			
Mean	10.3	4.69	5.97	12.3
S.D.	2.70	3.32	2.53	7.01

also observed in the current study as the mean production rates dropped 3-fold from 0.414 g/day · kg^{3/4} at birth to an average of 0.144 g/day · kg^{3/4} after birth (Table 2). The mean postbirth Hb production rate estimate of 0.144 g/day · kg^{3/4} (corresponding to an erythropoiesis rate of 3.84×10^9 RBCs/day · kg^{3/4}) is approximately half the production rate in healthy adults of 0.260 g/day · kg^{3/4} (6.3 g/day in a 70-kg adult) (Hoffman et al., 2005). This modest Hb production rate in these infants suggests that the production rate is suppressed. Specifically, the administration of RBC transfusions

to these infants, which prevents more severe anemia, may be limiting Hb production, particularly considering that adults under severe chronic anemic conditions are capable of increasing their Hb production rate 3- to 5-fold above normal (Hillman and Finch, 1967). The possible limiting effect of RBC transfusion on erythropoiesis is illustrated in Fig. 3A, where the plasma EPO concentrations (top) drop after each RBC transfusion. However, a similar pattern in the relationship between transfusions and plasma EPO concentrations was not observed in all subjects. For example, the representative subject displayed in Fig. 3B does not show any obvious relationship between RBC transfusions and plasma EPO concentrations. Either way, any effect of RBC transfusion on EPO is accounted for in the model because the cubic spline curve fitted to the plasma EPO concentrations acts as a forcing function. However, it is acknowledged that the model would be more physiologically complete if there was a negative feedback between Hb concentrations and EPO production.

The estimates of E_{max} and EC_{50} in Table 2 may be biased because they could only be determined in a subset of the

infants ($n = 6$) where the production rate was operating in the nonlinear (i.e., higher) plasma EPO concentration range. That is, in this subset of six infants the Hb production rate was approaching saturation, whereas in the other eight infants no production rate saturation was present in the observed plasma EPO concentration range. However, the mean estimates of $E_{\max}$ and EC_{50} suggest that the erythropoiesis or Hb production rate could be increased severalfold from of $0.144 \text{ g/day} \cdot \text{kg}^{3/4}$ of body weight up to $0.566 \text{ g/day} \cdot \text{kg}^{3/4}$ with only a relatively modest increases in the plasma EPO concentration, because the mean estimated EC_{50} was only 28.5 mU/ml. The increased plasma EPO concentrations and erythropoiesis rate could be achieved with exogenous administration of rHuEPO due to its identical amino acid sequence and biological equivalence to the natural hormone (Egrie et al., 1986). However, previous research has demonstrated limited success in the administration of rHuEPO to sufficiently increase erythropoiesis such that a clinically relevant reduction in RBC transfusions is achieved in preterm VLBW infants (Ohlsson and Aher, 2006). The exact reasons for the apparent limited efficacy of rHuEPO in infants are unknown, but they may be related to excessive phlebotomy blood loss, suboptimal dosing regimens, and insufficient understanding of the complex PDs of the response of erythrocytes to EPO. Alternatively, increased plasma EPO concentrations and erythropoiesis may also be achieved by allowing the Hct to fall lower before administration of a RBC transfusion(s). The increased hypoxia due to a lower Hct would probably increase endogenous EPO production and subsequently increase erythropoiesis.

The estimated 4-fold maximal increase in Hb production rate is consistent with the estimates in adults under severe chronic anemic conditions where 3- to 5-fold increase in Hb production rate occurs (Hillman and Finch, 1967). In addition, the mean maximal Hb production rate estimate of $0.566 \text{ g/day} \cdot \text{kg}^{3/4}$ was also similar to the estimated mean in utero Hb production rate from the current study of $0.414 \text{ g/day} \cdot \text{kg}^{3/4}$, which is a more hypoxic environment than that experienced after birth and thus may also represent a near-maximal Hb production rate. Similar to the postnatal fall in erythropoiesis experienced by these infants, the plasma EPO concentrations (Fig. 2) also fell after birth.

Previous RBC life span estimates based on ^{51}Cr -labeled RBCs from premature infants of 35 to 50 days (Brugnara and Platt, 2003) are similar to the results obtained in the current study of 65.8 days (Table 2). As expected, the estimated infant life spans were shorter than the typically referenced adult RBC life span of 120 days (Landaw, 1988; Brugnara and Platt, 2003). The estimated mean blood volume of 97.7 ml/kg in the first weeks of life (Table 2) is consistent with other measurements of blood volume in term and premature infants ranging from 89 to 110 ml/kg during the first 2 weeks of life (Sisson et al., 1959; Usher and Lind, 1965; Simon et al., 1998).

The number (Table 1) and rate of phlebotomy blood loss (Fig. 1) in these VLBW infants was extensive and contributes substantially to their anemia and RBC transfusion need. It was estimated that on average more than 50% of the Hb present at birth was removed during the first 30 days of life (Table 3). In addition, the mean amount of Hb removed was

greater than the estimated total amount of Hb produced and inputted into the circulation by these infants in the first 30 days of life. The transfused Hb amount was approximately twice the phlebotomy blood loss. Furthermore, as suggested by some subject's plasma EPO concentration-time profiles and discussed above, the administration of RBC transfusions may contribute to continued transfusion need by suppressing endogenous EPO production and the subsequent erythropoiesis. The effect of the estimated amount of blood loss ($38 \mu\text{l}$) added at each phlebotomy due to blood wiped away before capillary heel stick blood collection and clearing of blood from catheter lines at each indwelling catheter blood collection was minimal, as halving or doubling this estimated amount only changed the average blood loss by -6.07 or 12.1% , respectively.

The common goal with rHuEPO and other erythropoiesis-stimulating agent therapy in preterm VLBW infants is not just to reduce the number of RBC transfusions but also to eliminate them and thus the disease transmission and other risks associated with them (Ohlsson and Aher, 2006). With the common use of stored RBCs from a single donor, multiple RBC transfusions carry little additional risk of disease transmission than a single transfusion. Based on the estimated amount of Hb produced and the measured amount of Hb transfused (Table 3), an erythropoiesis-stimulating agent would need to increase the Hb production rate 2- to 3-fold to maintain Hb concentrations high enough to avoid the need for a RBC transfusion in the first 30 days of life under the current clinical practice guidelines used in the study. If the phlebotomy blood volume removed due to clinical testing could be substantially reduced and/or if the Hct percentage used in determining whether a transfusion is needed was decreased, then the Hb production rate needed to avoid a transfusion in these subjects would be less (Ohlsson and Aher, 2006).

Conclusions

In summary, a PD model that accounts comprehensively for phlebotomy losses and RBC transfusions was fitted to endogenous plasma EPO, Hb, RBC, and body weight profiles over time from 14 VLBW infants. Detailed recording of all blood removed from and administered to these infants and use of a Hb mass balance model allowed for a mathematically rigorous determination of the in vivo erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations under the dynamic and complex hematological conditions routinely experienced by VLBW infants in first 30 days of life. The estimated parameters of the PD Hb mass balance model were consistent with those in other published reports using direct measurement techniques, further supporting the utility of the proposed model. Future work with this model and parameter estimates, including direct measurements of blood volume and RBC life span in these subjects, will allow for an assessment of the potential to eliminate RBC transfusions in VLBW infants through administration of erythropoiesis-stimulating agents and/or changes in other clinical practices.

Appendix

Derivation of the Phlebotomy Correction Factor for the Differential Equation Output Terms

Let event A_j be the removal of RBCs (cells) or Hb by the j th phlebotomy and thus the complement, A_j^c , is the event that cells or Hb are not removed by the j th phlebotomy. Obviously, cells that entered the systemic circulation after the $k - 1$ phlebotomy cannot be affected by the phlebotomies conducted before the k th phlebotomy; thus, probability of removal by these phlebotomies is zero. Similarly, cells that exit the circulation due to the age-related cellular death before the $l + 1$ phlebotomy cannot be affected by phlebotomies after the l th phlebotomy, and the probability of removal by these phlebotomies is also zero. Therefore, only the effect of phlebotomies k through l on the differential equations need to be accounted, given that at least one phlebotomy was conducted between entry and exit (due to age-related cell death) of the cells from the circulation. The quantity of interest then is the probability that a cell is not removed by phlebotomies k through l , as this is the fraction of remaining cells that will exit via the age-related death through the appropriate differential equation term. This quantity can also be written as the probability of the intersection of events A_k^c through A_l^c [i.e., $P(A_k^c \cap A_{k+1}^c \cap \dots \cap A_l^c)$]. The conditional probability of not being removed by the j th phlebotomy is given by

$$\begin{aligned} P(A_k^c) &= F_{Pk} \\ P(A_{k+1}^c | A_k^c) &= F_{Pk+1} \\ P(A_{k+2}^c | A_k^c \cap A_{k+1}^c) &= F_{Pk+2} \\ &\vdots = \vdots \\ P(A_l^c | A_k^c \cap A_{k+1}^c \cap \dots \cap A_{l-1}^c) &= F_{Pl} \end{aligned}$$

where $P(\cdot)$ denotes the conditional probability. By rearrangement of Bayes theorem and substitution from above

$$\begin{aligned} P(A_k^c \cap A_{k+1}^c) &= P(A_{k+1}^c | A_k^c) \times P(A_k^c) = F_{Pk+1} \times F_{Pk} \\ P(A_k^c \cap A_{k+1}^c \cap A_{k+2}^c) &= P(A_{k+2}^c | A_k^c \cap A_{k+1}^c) \times P(A_k^c \cap A_{k+1}^c) \\ &= F_{Pk+2} \times F_{Pk+1} \times F_{Pk} \end{aligned}$$

which generalizes to

$$\begin{aligned} P(A_k^c \cap A_{k+1}^c \cap \dots \cap A_l^c) &= P(A_l^c | A_k^c \cap A_{k+1}^c \cap \dots \cap A_{l-1}^c) \\ &\quad \times P(A_k^c \cap A_{k+1}^c \cap \dots \cap A_{l-1}^c) \\ &= F_{Pl} \prod_{j=k}^{l-1} F_{Pj} \\ &= \prod_{j=k}^l F_{Pj} \end{aligned}$$

If no phlebotomies were performed between entry and exit (due to age-related cellular death) of the cells from the circulation, then no correction factor is needed, or equivalently the correction factor is equal to 1. This completes the derivation of the

phlebotomy correction factor for the differential equation output terms.

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Address correspondence to: Dr. Peter Veng-Pedersen, College of Pharmacy, The University of Iowa, 115 S. Grand Ave., Iowa City, IA 52242. E-mail: veng@uiowa.edu