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Biotin Labeling of Red Cells in the Measurement of Red Cell Volume in Preterm Infants

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ABSTRACT. Determination of circulating red cell volume (RCV) in anemic preterm infants is, in theory, a better indicator of transfusion needs than Hb concentration. Our study reports the results of RCV measurement using biotin labeling of red cells on 40 occasions in preterm infants of 25–34 wk gestation. In 20 infants, who had estimations made within 24 h of birth, the RCV varied between 17.7 and 66 mL/kg. Twenty measurements were made at a later age at the time of a blood transfusion. RCV values were between 13.1 and 41.5 mL/kg before transfusion. In 13 infants, RCV was determined simultaneously using two methods, biotin and dilution of autologous HbF with donor HbA at transfusion. There was no significant difference between the results of RCV estimations using these two methods. Our study demonstrates that biotin labeling is an effective method for determining RCV in preterm infants. (*Pediatr Res* 28: 199–202, 1990)

Abbreviations

Hct, hematocrit
RCV, red cell volume

The RCV represents the total volume of red cells in the circulation. It has been suggested that the RCV is a better guide to red cell transfusion requirements than Hb concentration (1–3), which has been shown to be a poor predictor of benefit from blood transfusion (4–6). After acute blood loss and in anemia of prematurity, the Hb concentration is poorly correlated with RCV; some infants have a very low RCV, yet maintain an adequate Hb concentration (1, 2, 7). RCV may also be a more rational physiologic indicator than Hb concentration of the oxygen carrying capacity of the blood in the whole circulation (3). Low RCV at birth is associated with birth asphyxia (8, 9), increasing severity of hyaline membrane disease (9, 10), and increased mortality (8, 10). RCV estimation has become a vital investigation in adults with polycythemias, some anemias, and the assessment of erythropoiesis (11).

Previously described methods for determining RCV include both techniques using the dilution principle after labeling of red cells with a tracer and indirect methods, which give a calculated RCV derived from plasma volume and Hct. Radioisotopes as tracers (11) are no longer acceptable in infants. Labeled albumin leaks in an unpredictable manner from the circulation in sick patients (12) and equilibrates with extravascular tissues (13). Therefore, determinations made with labeled albumin tend variably to overestimate plasma volume. Accurate calculation of RCV from plasma volume requires not only accurate plasma volume estimation but also a knowledge of the total body Hct. Although correction factors allow for the difference between venous and total body Hct (14, 15, 16), they are of quite unknown reliability in sick infants.

RCV can be estimated using dilution of autologous HbF at the time of a blood transfusion (2, 17). This is reliable provided that HbF estimation is accurate (*e.g.* alkali denaturation technique) (2) and at least 20% of the Hb in the circulation before transfusion is fetal. This technique cannot be used without a donor blood transfusion.

A new method has recently been described for determination of RCV in adults. Biotin is used to label autologous red cells, and their dilution is quantitated in a fluorescence-activated cell sorter using the fluorescein-streptavidin reaction (17). Biotin is not toxic in infants, even in pharmacologic doses (18).

We report here results of a study using biotin labeling of red cells for determination of RCV in preterm infants both within 24 h of birth and later at the time of transfusion.

MATERIALS AND METHODS

Infants. RCV determinations were made within 24 h of birth in 20 preterm infants, 13 male and seven female. Gestational ages at birth were 25–34 wk, birth wt 620–2400 g. Four of the infants were small for gestational age.

RCV estimations were made in relation to 20 transfusions at a mean age of 25 d (range 2–40 d) and mean wt of 1.5 kg (range 0.68–2.9 kg). Twelve infants were either being ventilated or receiving supplementary oxygen; the remaining eight were nursed in air at the time of transfusion.

The indications for red cell transfusions were as follows: *1*) Infants receiving assisted ventilation or those requiring a fraction of inspired oxygen of greater than 0.4: red cell transfusions were given if more than 9 mL/kg of blood had been sampled since birth or the previous transfusion, or if the Hct was less than 0.42 (Hb less than 14 g/dL). *2*) Infants requiring increased ambient oxygen but a fraction of inspired oxygen less than 0.4 within the first 2 wk of birth: transfusions were given if the Hct was less than 0.36 (Hb less than 12 g/dL) or if more than 9 mL/kg of blood had been sampled since birth or previous transfusion. *3*) Older infants, who were thought to be symptomatic, were given a transfusion if the Hct was less than 0.30 (Hb less than 10 g/dL), or if greater than 9 mL/kg of blood had been sampled since the previous transfusion. Asymptomatic infants received blood transfusion if the Hct was less than 0.27 (Hb less than 9 g/dL).

To validate the biotin technique in infants, RCV was determined simultaneously using both the present method and dilution of autologous HbF with donor HbA (2) in 13 infants receiving red cell transfusion.

In 18 infants studied just before transfusion, the posttransfusion RCV was calculated from the sum of the measured pretrans-

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fusion value and the volume of transfused red cells. In two cases studied just after transfusion, the pretransfusion RCV value was calculated by subtraction of the volume of red cells transfused.

Hb concentrations were measured on arterial or venous blood samples with each determination of RCV. The amount of placento-fetal transfusion at birth varied because the timing of cord clamping was uncontrolled.

Ethical approval for this study was obtained from the Hospital Ethics Committee and informed parental consent was obtained before each measurement of RCV.

Determination of RCV. Biotin labeling of autologous red cells was used for determination of RCV by a dilution technique. The method was modified from that of Cavill *et al.* (17). Heparinized blood (0.5 mL) from the infant was placed in a sterile container, 5 mL of normal saline added, and the sample centrifuged at 1100 rpm for 5 min. The red cells were then removed and resuspended in 2 mL of normal saline. Some of the resuspended sample (0.3 mL) was kept for a cell count and to form the negative control. The remainder of the sample was incubated for 15 min at room temperature, with constant agitation for the first 5 min, with 0.35 pg biotin per cell (long chain, water soluble biotin, NHS-LC-Biotin Sulfosuccinimidyl 6-(biotinamide) Hexanoate, Pierce Chemical Company, Rockford, IL). The biotin had been reconstituted by dissolving in sterile normal saline and filtering through a 0.2 μm filter. After incubation, the cells were washed twice and resuspended to 1.3 mL. Some of this suspension (0.3 mL) was kept for a cell count and to form the positive control. The remainder was reinjected into the infant.

At 10 and 30 min after reinjection, 0.3 mL blood was taken from the infant. A cell count was performed on part of each sample. Ten million cells of duplicates of the negative control, positive control, and 10- and 30-min samples were then resuspended in 1 mL cold PBS. Ten μL of fluorescein-streptavidin (Amersham International plc. Amersham, Buckinghamshire, UK) were added to each of the eight tubes; incubation was over 30 min at 2–4°C, with agitation every 10 min. After incubation, the cells were washed twice and resuspended in 1 mL particle-free saline for counting. One million cells from each of the eight tubes were counted on a fluorescence-activated cell sorter (Becton-Dickinson "FACS 440"). The cells were excited at a wavelength of 488 nm with an argon laser and analyzed at 535 nm. The labeled cells were readily distinguishable from the unlabeled cells and, therefore, the proportion of cells fluorescing could be determined. The negative control was used to give the background fluorescence (normally <0.03%), which was subtracted from the 10- and 30-min samples. The positive control confirmed effective binding (normally ~99%). The RCV can be calculated from the equation:

$$\text{RCV (mL/kg)} = \frac{\text{vol inject} \times \text{RCC} \times \%\text{ positive control} \times \text{MCV}}{\%\text{ positive samples} \times 1000 \times \text{wt (kg)}}$$

where vol inject is the volume of blood reinjected into the infant, RCC is the red cell count of the reinjected sample, % positive control is the % positively labeled cells in the positive control, MCV is the mean cell volume (fL) (determined on an unlabeled sample) needed to convert a cell count into a volume, and % positive samples is the mean of the proportion of positive cells in the 10- and 30-min samples minus negative control.

RESULTS

RCV values within 24 h of birth (including allowance for sampling blood losses), as shown in Figures 1 and 2, ranged from 17.7–66.5 mL/kg, mean 34.2 mL/kg. In one growth-retarded infant of 33 wk gestation, the RCV was 66.5 mL and the venous Hct was 0.63. The small-for-gestational-age infants as a group had a mean RCV of 44.2 mL/kg. RCV values calculated from the samples obtained 10 min after reinjection showed no significant difference from those determined from the samples taken at 30 min, implying adequate mixing at the earlier time. Mean Apgar scores at 5 min in infants with RCV < 30 mL/kg were 7.3, with RCV 30–40 mL/kg, 7.7, and with RCV > 40 mL/kg, 8.8; the differences were not statistically significant. There was no significant correlation with gestational age although the two infants of shortest gestation had the lowest RCV values. Table 1 shows RCV values in relation to gestational age at birth.

Three of the infants subsequently died, two from complica-

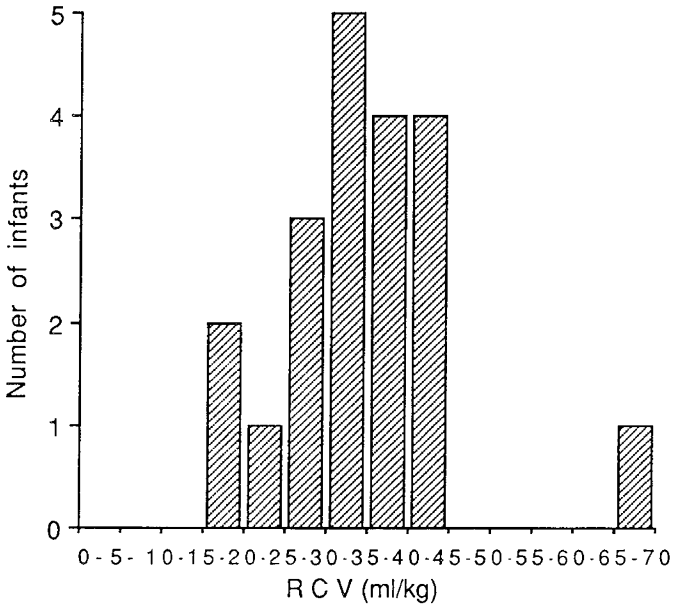

Fig. 1. Results of RCV determination within 24 h of birth in 20 preterm infants.

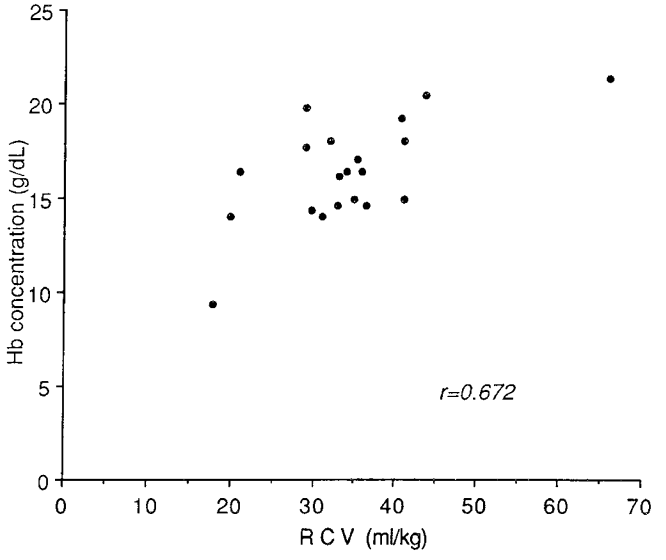

Fig. 2. Relationship between Hb concentration and RCV within 24 h of birth in 20 preterm infants.

Table 1. *RCV determination within 24 h of birth in relation to gestational age*

Gestational age (wk)	*n*	RCV (mL/kg) Mean	SD
25–27	3	22.2	6.1
28–30	6	35.3	3.9
31–32	4	41.9	17.3
33–34	7	34.1	7.2

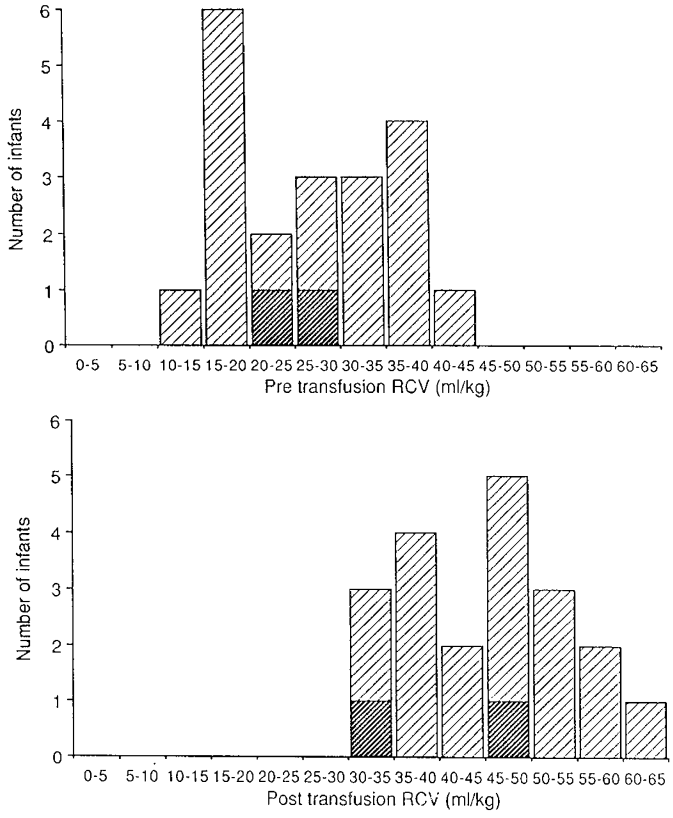

Fig. 3. Results of RCV determination at the time of red cell transfusion in 20 preterm infants. RCV was measured in 18 cases before and in two cases after transfusion, the corresponding post- or pretransfusion volumes were calculated by adding or subtracting, respectively, the volume of the transfused cells. These cases are indicated by *dark hatches.*

Table 2. *RCV determinations in relation to blood transfusion at mean age of 25 d (range 1–90 d)*

		Pretransfusion RCV (mL/kg)	
	n	Mean	SD
Gestational age at birth (wk)			
26–27	8	32.4	6.9
28–29	7	23.7	7.4
30–31	3	27.8	11.4
32–33	2	16.7	0.1
Postconceptual age at transfusion (wk)			
26–27	5	28.4	8
28–29	4	30.6	9.1
30–31	4	32.3	9.1
32–33	2	18	2.3
34–35	1	18	
36–37	2	25.3	12.8
38–39	2	14.9	2.6

tions of prematurity (intraventricular hemorrhage and severe idiopathic respiratory distress syndrome) and one from severe cyanotic heart disease. The two infants who died from complications of prematurity had the lowest RCV values, 17.7 and 19.8 mL/kg.

Hb concentration and RCV within 24 h of birth were correlated, but not very closely ($r = 0.678$). The correlation between Hct and RCV in older infants being transfused ($r = 0.698$) was similar to that at birth. Because of insufficient data in severe anemia, its effect on the Hct-RCV relationship could not be examined.

RCV values determined before transfusions are shown in Figure 3 and ranged from 13–41 mL/kg, median 26.3 mL/kg; the corresponding Hb concentrations were 7.9–17.7 g/dL, median 12.0 g/dL. Posttransfusion RCV ranged from 32–60 mL/kg, median 44.4 mL/kg; corresponding Hb concentrations were 15.2–20.7 g/dL, median 17.8 g/dL. Table 2 shows infants' RCV values at the time of transfusion in relation to the infants' gestational age at birth and their postconceptual age (*i.e.* gestational age at birth plus postnatal age).

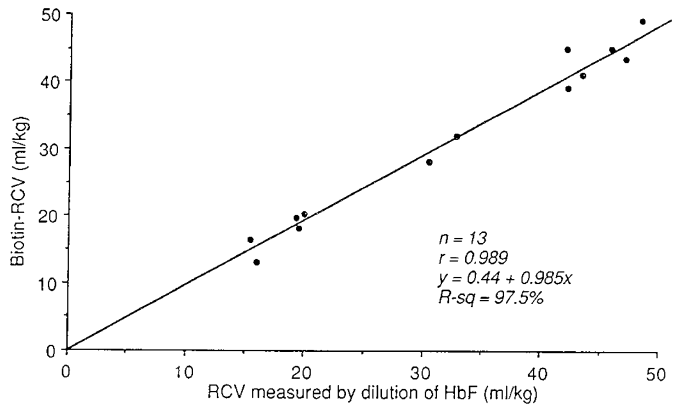

Fig. 4. Relationship between RCV determined by two independent methods: dilution of HbF and biotin labeling.

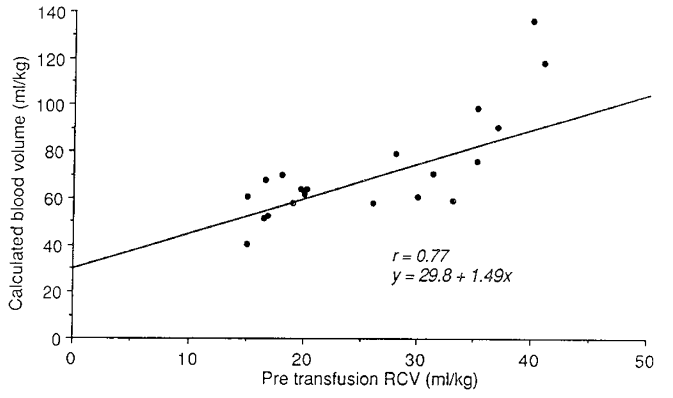

Fig. 5. Relationship between RCV and calculated blood volume before red cell transfusion in 20 preterm infants. No correction factors for total body hematocrit:venous hematocrit have been applied.

RCV estimations were repeated in some infants. Total clearing of labeled cells occurred in approximately 3–4 wk. If repeat measurements were made before this time had elapsed, the negative control (background fluorescence) was higher, but RCV estimation was still possible.

As shown in Figure 4, results of RCV estimated simultaneously using biotin and dilution of HbF by donor HbA are similar. Neither the ranked pair differences nor the median of the two estimations were significantly different ($p > 0.05$).

In infants needing transfusion, the pretransfusion blood volume was estimated using the RCV and Hct. Correction factors for venous to total body Hct have not been applied. The results are shown on Figure 5. There appears to be an association between a low RCV and a low circulating blood volume thus calculated, providing the ratio of total body Hct to venous Hct remains constant and is unity.

DISCUSSION

This study was designed to develop and evaluate biotin tracer labeling of red cells for measuring RCV in newborn infants. Biotin as a tracer has been validated in adults by ^{51}Cr determination of RCV made simultaneously with biotin estimations (17). Our study validates the measurement in infants by comparing RCV determination made simultaneously by both biotin

labeling and dilution of fetal Hb by donor adult Hb at transfusion. Total clearing of labeled cells appeared to take 3–4 wk, which is several times longer than in adults. It is not clear why there should be a difference between infants and adults. Both the site of binding of biotin to the surface of the red cell and the mechanism of clearance of labeled red cells are unknown. This delay in clearing labeled red cells does not preclude repeated measurements; several infants have had serial estimations of RCV made at intervals of 4–7 d or more without difficulty. The background fluorescence in the negative controls in these infants is higher, but allowance can be made for this by subtracting the background fluorescence from the samples taken at 10 and 30 min.

Estimations of RCV were made in infants receiving parenteral nutrition or enteral breast or formula milk. The technical problems encountered in adults who had consumed avidin-containing foods such as eggs (17) did not arise in this study.

The values for RCV in 20 preterm infants accorded with those of others (2, 6, 7, 9, 16, 19, 20). Our relatively small numbers and bias toward extreme prematurity in the infants with the lowest RCV limited the opportunity of analysis of the outcome in relation to RCV. It is, however, of interest to note that the two infants with the lowest RCV were those who died from complications of prematurity. Our study supports the association between low RCV and birth asphyxia (8, 19).

The varied relationship between RCV and Hb concentration (or Hct) has been noted before (1, 2, 7). Our findings emphasize this important point both at birth and pretransfusion. As previously noted, the RCV in anemic infants may be very low, yet the Hb concentration may be well maintained (1, 2, 7, 8). In our study, seven out of 20 RCV values before transfusion were less than 20 mL/kg, yet in only one baby was the corresponding Hb concentration less than 10 g/dL. Thus, further evidence is provided that the Hb concentration (or Hct) may mask a serious deficiency of circulating red cells. The overlap of pre- and post-transfusion RCV values and Hb concentrations is not surprising given the heterogeneity of clinical problems and decisions on indications for red cell transfusion. This technique may permit rational prescribing of red cells based on RCV to endow the infant with a RCV sufficient to meet anticipated oxygen requirements (3). This cannot be accurately predicted from the Hct (1).

The circulating blood volume was calculated indirectly from the RCV and the central Hct. This may not be accurate. It is important to note that without reliable plasma volume estimation made simultaneously with RCV determination, total blood volume cannot be accurately established. In view of the unreliability of albumin as a circulating plasma label (12, 13), the true total body to venous Hct ratio is unknown in sick, preterm infants. Although this ratio has been assumed to be linear throughout the whole RCV range, this might not be valid. It has been suggested that the blood volume to Hct ratio in sick and anemic infants is lower than in healthy infants (21, 22). As noted by Mollison (23), in sick patients the volume of distribution of small molecules such as albumin unpredictably exceeds the volume of distribution of the red cells, rendering inaccurate the estimation of circulating plasma volume using labeled albumin. Variably increased capillary permeability is at the root of this problem (13, 23). In such patients, accurate means of estimating true circulating plasma volume have eluded investigators.

Knowledge of the whole body Hct is important in accurate assessment of polycythemia, but arterial blood Hct may be more relevant as a representation of oxygen transport capacity in the circulation. The result of this calculation of blood volume implies a reduction in blood volume associated with a deficiency of circulating red cells. This could offer the advantage of maintaining the Hct and therefore systemic oxygen transport at a given cardiac output, but at the expense of poor perfusion of some parts of the circulation such as the splanchnic bed, lung, and skin.

Our study demonstrates that biotin labeling of red cells is a safe and effective technique for determining the RCV in preterm infants. The technique requires a flow cytometer and takes about 4 h to perform, but is likely to be of special value in selected sick infants in whom appropriate management depends on the quantitation of abnormalities in RCV. In adults, determination of RCV is essential in the investigation of polycythemia and some anemias and in the assessment of erythropoiesis. Determination of RCV using biotin as a tracer should permit these pathologic states to be investigated in the newborn, leading to improvements in the diagnosis and clarification of their pathology.

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