Morphology and Filterability of Red Blood Cells in Neonatal and Adult Rats

K. GUNNAR ENGSTRÖM AND LARS OHLSSON

Department of Histology and Cell Biology, University of Umeå, S-901 87 Umeå, Sweden

ABSTRACT. Red blood cell (RBC) deformability in rats of various ages was assessed by filtration (3 μm Nuclepore membranes). Neonatal rat RBC (1 d old) had lower filterability, both in terms of RBC incremental volume (9.97 ± 1.85 versus 0.33 ± 0.28 nL at 180 d of age, mean $\pm$ SD, $p < 0.001$) and the number of filter clogging particles (25.7 ± 3.1 versus 18.9 ± 3.4 RBC $\times 10^3/\text{s}$, $p < 0.001$). The lower filterability correlated with a larger RBC volume (169 ± 12.6 versus 69 ± 3.2 μm^3 , $p < 0.001$) and with a wider minimum cylindrical diameter (3.87 ± 0.15 versus 2.84 ± 0.05 μm , $p < 0.001$). Almost all of the neonatal RBC had a minimum cylindrical diameter exceeding the 3 μm nominal pore size of the filters. The calculated resistance to initial folding was also significantly greater, as indicated by a static bending analysis of initial deformation. However, when the larger size of neonatal RBC was taken into consideration, and thus their greater projected area on which forces are applied, they appear to be at least as deformable as the adult type RBC. This finding may explain the contradiction between RBC filtration experiments and other approaches based on RBC deformations in shear flow, which have been unable to detect a hampered flexibility of neonatal RBC. In view of the more pronounced differences between neonatal and adult RBC in rats than in human subjects, the rat is an interesting model for studying this physiologic phenomenon in newborns. (*Pediatr Res* 27: 220–226, 1990)

Abbreviations

MCD, minimum cylindrical diameter
MCH, mean corpuscular hemoglobin
MCHC, mean corpuscular hemoglobin concentration
MCV, mean corpuscular volume
RBC, red blood cell
RBCiv, red blood cell incremental volume
WBC, white blood cell
SAI, surface area index
SWI, swelling index

Human neonatal RBC are less filterable compared with adult RBC (1). The filterability is also known to be lower in preterm than in term neonates (2). The diminished ability to pass through narrow pores may cause sequestration and shorter life span of neonatal RBC (3, 4) and may also contribute to serious circulatory dysfunctions, e.g. congestive heart failure, respiratory dis-

tress, cyanosis, cerebral infarction, and necrotizing enterocolitis (5–9).

RBC deformability is one of the major determinants of blood rheology and is influenced by 1) cellular shape, of which the area-to-vol ratio is usually emphasized, 2) cytoplasmic and membrane viscosity, and 3) RBC aggregation. In neonates, RBC are generally large with a decrease in membrane area-to-vol ratio (10), which is the most likely explanation for their greater resistance to pore transit when assessed by filtration or micropipette aspiration (1, 2, 11). However, neither cytoplasmic viscosity nor membrane mechanical properties differ notably between neonatal and adult RBC (11, 12).

Viscometry, rheoscopic measurements (4), and ektacytometry (13) have, in contrast to filtration experiments, shown no differences in deformability between neonatal and adult RBC. Filterability measurements are more sensitive to RBC shape than cytoplasmic viscosity or membrane rigidity, which is thought to explain the discrepancy between this and the other approaches more specifically reflecting cell viscosity. In the microcirculation, however, capillary diameters may be significantly smaller than the diameters of the RBC. In fact, the thinnest capillaries appear to be very close to the limit of deformation of circulating RBC (14). This encourages the use of filtration in assessing RBC deformability.

In view of the necessity of RBC flexibility, the lesser ability of neonatal RBC to deform needs further investigation to explore the mechanisms behind circulatory dysfunctions in the newborn. So far, all studies have concerned human RBC. In our study, rats of ages from 1 to 180 d were analyzed. RBC deformability was measured by a filtration technique making it possible to evaluate both the pore transit resistance and the proportion of filter clogging cells. Cross-sections of individual RBC were measured and deformability expressed in terms of area-to-vol ratio, SAI (the relative excess of membrane area beyond that required to enclose the cellular volume), and SWI (the relative swelling to become a sphere of same membrane area), MCD (the smallest possible diameter of a tube-shaped RBC), but also by means of a static bending analysis of initial folding (15–18).

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats were purchased from Alab Laboratories AB (Stockholm, Sweden). Three different age groups were used. Neonatal rats were obtained together with their mothers within 24 h from delivery. One rat from each of six litters was cardiopunctured at 1, 10, and 20 d of age. One group of six rats was studied at 40 and 70 d. Another group of six rats was followed between 100 and 180 d of age. Rats were kept under controlled conditions of constant temperature (22–24°C), humidity (55–65%), and illumination from 0600 to 1800 h.

Blood sampling. Neonatal and young rats (1–20 d of age) were anaesthetized using intraperitoneal pentobarbital, 100 mg/kg. Blood was obtained from the right heart ventricle by means of a mouth-operated collecting tube, in which EDTA had been added

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Correspondence and reprint requests K. Gunnar Engström, Department of Histology and Cell Biology, University of Umeå, S-901 87, Umeå, Sweden.

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as a solid to give a final concentration of approximately 5 mmol/L whole blood. Whole blood, 44.7 μ L, for electronic measurements, was aspirated into a calibrated pipette and suspended in 10 mL Isotone II medium (Unopette microcollection system, Becton Dickinson, Rutherford, NJ). Another 5 μ L of blood were diluted in 1 mL RBC suspension media for morphologic examinations, whereas the rest was aspirated into a thin, 12-cm long polyethylene capillary tube (code 19-0040-01, Pharmacia, Uppsala, Sweden). The capillary tube was sealed at one end by gently heating it over a flame. Each batch of tubes had been carefully calibrated by weighing the water contained by a certain length. The variability in displacement within each batch is negligible (19). The tubes with blood were centrifuged at $1500 \times g$ for 15 min (4°C) supported by a rigid holder. The microhematocrit was recorded and a length corresponding to 25.0 μ L of packed RBC was cut from the mid-section of the RBC column and suspended in 50 mL of buffer to yield a hematocrit of 0.05%.

In adult, unanesthetized rats (40–180 d of age), blood was obtained from the tail tip. After a few min warming of the tail with an infrared lamp, vessels dilated and blood was easily sampled. Blood was collected into microfuge tubes, which contained EDTA, and was processed as explained above.

RBC was incubated at a low concentration of bovine serum albumin at room temperature for 1 h before filtration and morphologic measurements, to minimize stomatocytic shape transformation (Engström KG, Täljedal I-B, unpublished data).

RBC suspension media. The RBC suspension media was a Hepes-buffered Krebs-Ringer solution of a calculated 300 mosmol/L supplemented with 0.2 g/L BSA (fraction V, Pentex, Miles Scientific, Naperville, IL) and 5 mmol/L glucose. The pH was adjusted to 7.40 using NaOH. The medium was prefiltered through 0.8 μ m Nuclepore membranes before use.

RBC and WBC counting. In whole blood, the RBC count, Hb, MCV, MCH, MCHC, WBC count, and platelet count were measured by means of a Coulter counter (Model S-plus, Coulter Electronics, Inc., Hialeah, FL). The concentration of resuspended RBC used for filtration experiments, as well as the occurrence of nucleated cells in suspension, were measured in a counting chamber by light microscopy. Nucleated cells were stained with Türk's reagent (0.02% gentianviolet B in a 6.2% acetic acid solution).

Measurements of RBC morphology. A special measuring chamber for RBC morphology was made from cover-slips on a microscope slide. Into a rectangular compartment, measuring approximately $18 \times 10 \times 0.17$ mm, a drop of suspended RBC was introduced and the openings sealed with immersion oil. RBC were allowed to sediment with the chamber held upside down. After 2 min, the chamber was turned right side up and mounted in the microscope. A large proportion of the previously settled RBC were then hanging from the upper glass surface. The RBC diametrical cross-sections were focused on using bright field illumination (Carl Zeiss $\times 100/1.25$ oil objective lens, Oberkochen, FRG). A digitizing table with light-spot cursor (MOP-Videoplan, Kontron Bildanalyse GmbH, Munich, FRG) was optically superimposed on the microscopic image, and the RBC diameter (D), maximum toroidal thickness (T), and central thickness (C) were measured. A thin diffraction band surrounded the RBC cross-sectional profile due to differences in refractive indices between RBC and medium. The uniform shell rule was therefore applied during measurements (20). The method is consistent with that described in detail in a previous publication (17).

RBC profile calculations. The RBC measurements (D, T, and C) were used to compute the membrane area and volume with an equation expressing the RBC cross sectional curvature (17, 18). MCD, SAI, and SWI are derived from area and volume according to:

$$0 = -\pi(MCD)^3/12 + A(MCD)/4 - V \quad (1)$$

$$SAI = 0.207 A V^{-2/3} \quad (2)$$

$$SWI = 0.094 A^{3/2} V^{-1} \quad (3)$$

Calculations of RBC bending mechanics. It is possible to express mathematically the mechanical resistance to initial bending deformation of the RBC corpuscle by means of a section modulus value. The section modulus only considers the RBC shape and size and not the membrane mechanical properties as such, e.g. elastic shear, dilation, or bending moduli, or their interrelations. The section modulus refers to the entire RBC corpuscle, built up by a 0.01- μ m thick membrane shell with linearly elastic behavior (21).

The corpuscle section modulus is further analyzed by means of a static bending model, in which the RBC is stressed by a pressure difference between the two sides of the cell (here set to a fictitious pressure of 1 kPa). The pressure load builds up a bending moment and hence membrane tensions, both having maxima at the RBC diametrical cross-section. The diametrical cross-section is therefore crucial for the initiation of deformation and is calculated as representative for the whole corpuscle (15, 18). The higher the tension, the more deformable is the RBC. The tension is calculated by division of bending moment with section modulus. This method of theoretical analysis may be valuable in evaluating the influence of size and shape only on the RBC deformability (15–18).

Measurements of RBC filterability. RBC suspensions were filtered through 3- μ m pore size Nuclepore polycarbonate membranes (diameter 13 mm, lot 62 EO B25 or 62 A5 C30 with an average pore density of 1.81×10^6 pores/cm²) driven by a constant hydrostatic pressure of 600 Pa. The filtration rate was measured by collecting the filtrate on a digital balance (Sartorius 3716, Sartorius Werke GmbH, Göttingen, FRG) and the weight recorded every 5 s during a 1-min filtration period. Before RBC filtration, each filter was calibrated by measuring the filtration rate for cell-free medium.

The ability of RBC to pass the filter pores was evaluated using a regression analysis of the filtration rate. The starting point of regression was taken to be 10 s, at which time all pores of the membrane were interacting with RBC as calculated from the average RBC particle concentration and the number of pores. At that time, 10 s, the filtration rate was stable and declined linearly owing to filter clogging by rigid RBC. To obtain the initial RBC filtration rate (FRBC) the regression line was extrapolated to 10 s in real time of the 60-s filtration period. The RBCiv was then calculated as:

$$RBC_{iv} = (F_m/F_{RBC} - 1)/RBC_{count} \quad (4)$$

This equation expresses the relative decrease in filtration rate due to RBC compared with the filtration rate of medium alone (F_m) and with respect to the concentration of RBC in the suspension (RBC count). Mathematically, the RBC incremental volume (L/RBC) is the volume of medium that is hindered from passing a narrow pore owing to the presence of an RBC within it.

The flow rate declines with time of filtration because of clogging particles (CP). These particles can be evaluated by relating the slope, k (filtration rate as a function of filtration time), and the average number of filterable pores (N_p , 1.15×10^6 pores) to the initial filtration rate, FRBC.

$$CP = N_p k/FRBC \quad (5)$$

The clogging particles reflect the frequency with which filter pores are plugged (RBC/s). The present calculations are in accordance with those described by Matrai *et al.* (22), except that filtration rate is here made a function of time instead of volume.

Statistical analyses. Results are given as mean values $\pm$ SD. Unpaired Student's *t* test was used to evaluate differences between neonatal (1 d old) and adult rats (180 d old, if not otherwise stated). Correction was made for unequal variance between groups.

RESULTS

Body wt gain declined from an average of 1.65 g/d in the neonatal period (d 1 to 10) to 0.54 g/d in the adult (d 100 to 181). At 181 d of age body wt was close to 300 g.

In Figure 1a-f, the micro-hematocrit, electronically measured whole blood Hb, RBC count, MCV, MCH, and MCHC are shown. The micro-hematocrit and hemoglobin in neonatal rats were not significantly different from those in the adult, but showed distinct minima around d 20 ($p < 0.001$ versus d 1 or 40). The RBC count increased successively and reached the adult level at 70 d of age ($p < 0.001$ versus d 1), whereas the MCV showed a pronounced decrease from the neonatal ($142 \pm 7.83 \mu\text{m}^3$) to the adult period ($56.6 \pm 1.59 \mu\text{m}^3$ between 20 and 180 d, $p < 0.001$). The MCH decreased ($p < 0.001$) in parallel with the MCV owing to a relatively constant MCHC.

In 10 d the platelet count increased from $468 \pm 53 \times 10^9/\text{L}$ in the 1-d old rat to the adult level of $843 \pm 130 \times 10^9/\text{L}$. WBC

decreased steeply to approximately half of the concentration in neonates. However, this Coulter measurement is likely also to include nucleated RBC, which were found to constitute $54 \pm 6.8\%$ of the total number of nucleated cells at 1 d of age. At 10 and 100 d of age, the fraction was 0.91 ± 0.64 or $0.21 \pm 0.33\%$, respectively. The recalculated true WBC count does not vary notably with age and is on the order of $8 \times 10^9/\text{L}$. On d 1, $96 \pm 1.6\%$ of the total number of nucleated cells were nucleated RBC in the RBC filtration suspension, a figure that fell to $2.2 \pm 1.9\%$ within 10 d. The relative proportion of nucleated RBC to the total number of RBC in the resuspension was about 0.05% in the 1-d-old rat and 0.00014% in the adult rat.

The measured diameter, toroidal (maximum) thickness, and central (minimum) thickness, calculated membrane area, and RBC volume are shown in Figure 2a-e. The RBC size decreased significantly ($p < 0.001$) to the adult levels, by 20 d for toroidal thickness, and by 70 d for diameter, membrane area, and volume. Central thickness showed a minimum at 20 d of age ($p < 0.001$

Fig. 1. a, micro-hematocrit; b, electronically determined Hb concentration; c, RBC count; d, MCV; e, MCH; and f, MCHC as a function of age. Mean $\pm$ SD in three groups of six rats each (1–20, 40–70, and 100–180 d of age).

Fig. 2. *a*, measured RBC diameter; *b*, toroidal thickness; *c*, central thickness; *d*, calculated membrane area; and *e*, volume as a function of age. Mean $\pm$ SD in three groups of six rats each (1–20, 40–70, and 100–180 d of age).

versus d 1 and 180) and then increased in the adult rat to a level about 75% less than ($p < 0.001$) that seen in the neonates.

Figure 3*a–f* show the deformability indices. These parameters were calculated from the morphologic measurements in Figure 2*a–c*. The area-to-vol ratio increased about 40% ($p < 0.001$) between 1 and 20 d of age to the adult level. SAI showed a corresponding increase ($p < 0.001$) between 1 and 20 d of age but then regained the neonatal level within 70 d. An almost identical curve was seen for the SWI (not shown). The proportion of RBC exceeding $3 \mu\text{m}$ in MCD, and section modulus, the calculated RBC resistance to deformation, decreased to a minimum at 20 d. However, the RBC membrane tension indicated a greater deformability for neonatal and young RBC, with a maximum at 20 d of age, but then a fall to the adult and less deformable level (Figure 3*f*). In other words, a neonatal RBC would need only 75% of the pressure load for an adult type RBC to obtain the same deformative membrane tension.

RBC filterability data are shown in Figure 4. RBCiv fell significantly ($p < 0.001$) between 1 and 10 d of age, whereas the filter clogging particles decreased more gradually ($p < 0.001$ between d 1 and 70). The apparent increase in clogging particles between 70 and 100 d of age reflects a new lot of Nuclepore filters, (see Materials and Methods).

DISCUSSION

Our data on neonatal rat RBC are, in principle, in agreement with previous reports on human neonates. The most obvious difference is that the variations in most RBC parameters between neonatal and adult RBC are more pronounced in rats than in humans. Thus, the mean RBC vol was about 2.5 times more in neonatal than in adult rats, but about 1.2 times greater for human RBCs (4, 23).

The concentration of RBC and Hb in whole blood is reflected

Fig. 3. Calculated *a*, area-to-volume ratio; *b*, SAI; *c*, MCD; *d*, proportion of RBC exceeding 3 μm in MCD; *e*, corpuscle section modulus (high section modulus indicates large resistance to whole cell folding); and *f*, membrane tension induced by a certain pressure load (high membrane tension indicates low resistance to membrane deformation) on the RBC as a function of age. Mean $\pm$ SD in three groups of six rats each (1–20, 40–70, and 100–180 d of age).

by the MCV, RBC particle concentration, and MCHC. Hematocrit and Hb decreased to a minimum around 20 d of age, probably owing to a sequestration of macrocytic neonatal RBC and the insufficient replacement of adult type, smaller RBC. However, the expansion of the vascular volume during growth is also to be considered. An approximate extrapolation of the almost linear fall in hematocrit and Hb would indicate a depletion of neonatal RBC within about 45 d, which is below the average life span of adult rat RBC, thought to be about 60 d (24). The life span for human neonatal RBC is 60 to 80 d as compared to 120 d for adult RBC (25).

The RBC size decreased substantially as the rat became adult. However, from a minimum at around 20 d of age the central thickness reached a new maximum at 100 d, although significantly below the level seen in the 1-d-old rats. In contrast to human neonates (10, 11), RBC maximum thickness (toroidal

thickness) was markedly greater in neonatal than in adult rat RBC. In the present study, observing RBC cross-sectional profiles, direct thickness measurements were made instead of approximations calculated from RBC vol and diameter (see Ref. 11, equation 3), and these may therefore be more accurate.

The MCD decreased steeply during the first 20 d of age. MCD reflects the final deformation into a tube-shaped RBC and is of interest because it gives an estimate of the ability of the RBC to pass narrow pores, *e.g.* in the microcirculation or in a filtration test. Therefore, the proportion of RBCs exceeding 3 μm in MCD (*i.e.* the nominal pore size of the Nuclepore membranes used for filtration) was also calculated. Close to 100% of the neonatal RBC would apparently plug the pores of the filter, a figure that diminishes to about 25–30% in adult rats. However, the SAI and SWI indicate a maximum membrane excess for rats of 20 d of age, whereas neonatal and adult rats are identical from this point

Fig. 4. *a*, RBC incremental volume (RBCiv, high RBCiv indicates increased resistance to filtration) and *b*, filter clogging particles as a function of age. The apparent increase in clogging particles between 70 and 100 d of age reflects a new lot of Nuclepore filters, also seen for RBCiv, although more vaguely (see Materials and Methods). Mean $\pm$ SD in three groups of 6 rats each (1–20, 40–70, and 100–180 d of age).

of view. This similarity between neonatal and adult RBC has been demonstrated for human neonates as well (10). The exact interpretation of these geometric indices in terms of RBC deformation is not clear-cut, but they may give a hint as to the general RBC deformability because of the basic need for excess membrane area (10).

The present morphologic method not only gives the correct thickness measurements on RBC cross-sectional profiles but also allows further calculations of RBC deformability as predicted by a static bending analysis of initial folding (15–18). The resistance to fold a neonatal corpuscle was almost twice that for an adult RBC. Interestingly when the projected cell area (the area on which the deformative pressure is loaded) and the distance between points of support during deformation are considered, the membrane tension induced by a given pressure load became significantly greater in neonatal than in adult RBC. Consequently, neonatal RBC may be more easily folded during the initial step of deformation than adult type RBC. This finding may help in explaining the discrepancy between filterability methods and other rheological approaches used to measure neonatal RBC deformability. Under shear forces in a rheoscope the projected area of the RBC must be assumed to play an important role for the cell deformation. At present, the static

bending model seems to be the only method that incorporates RBC diameter in the calculations of deformability.

The analysis of RBC filterability clearly indicated a lower deformability of neonatal RBC, both in terms of RBCiv and filter clogging particles. In addition to RBC shape, RBCiv is sensitive to the viscosity of the cytoplasm and/or the membrane. Inasmuch as the MCHC did not vary notably between neonatal and adult RBC, differences in Hb viscosity probably do not contribute to the diminished filterability of neonatal RBC. Fetal Hb does not seem to alter intracellular viscosity (1). Membrane mechanical properties are also unlikely to explain any differences in deformability, because membrane lipid composition is essentially the same for neonatal and adult RBC (26), as are lipid viscosity (12) and membrane elastic moduli (11).

The clogging particles more specifically reflect RBC shape and their tendency to plug the filter pores. The flow of RBC suspensions in the 1-d-old rat almost stopped after an average of 0.3 mL. The number of suspended RBC contained in this volume corresponds closely to the number of pores of the membrane, indicating almost total plugging. Inasmuch as close to 100% of neonatal RBC have an MCD above $3.0 \mu\text{m}$, it must be concluded that rigid and large RBC clog the filter pores and not contaminating nucleated cells, as is often claimed (27). Nucleated cells are also largely eliminated during the preparation of the RBC suspension (19). Furthermore, in a standard experiment only about 15 RBC will pass each filter pore, which makes the occurrence of nucleated RBC in the 1-d-old rats, 0.05%, and contaminating WBC, 0.002%, unlikely. RBCiv is not sensitive to nucleated cells either, because being a derivative of the initial RBC filtration rate is not affected by plugging cells of any sort.

Calculations indicate a maximum RBC deformability already at 20 d of age in terms of low MCD and section modulus, and high SAI, SWI, and membrane tension (Fig. 3). As suggested by the changes of hematocrit and Hb, it is likely that rigid neonatal RBC are sequestered at that time, and a pool of young, newly formed and highly deformable, adult type RBC occurs in the circulation. The aging of these adult type RBC is then observed in terms of a diminishing RBC diameter, membrane area, and volume, and an increasing central thickness (Fig. 2). These alterations are well-known attributes for aging of normal RBC (15), but should not be confused with differences between neonatal and adult RBC.

Our study shows that neonatal rat RBC are more resistant to pore transit, most likely because of their greater size. The impaired filterability through narrow pores may contribute to the shorter life span of neonatal RBC and to rheologic dysfunctions in newborns. However, a static bending analysis of initial deformation emphasizes that the neonatal RBC are not necessarily less deformable than those of adults, if their greater projected area is taken into consideration. This finding may explain the reported discrepancies between RBC filtration methods, on the one hand, and viscometry, rheoscopic, or ektacytometric approaches, on the other, in evaluating the deformability of neonatal RBC.

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