0031-3998/93/3405-0688$03.00/0
PEDIATRIC RESEARCH

Vol. 34, No. 5, 1993
Printed in U.S.A.

Mechanical and Geometrical Properties of Density-Separated Neonatal and Adult Erythrocytes

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ABSTRACT. Neonatal red blood cells (RBC) show large variations in size, density, and deformability, with a relatively high percentage of neonatal RBC being extremely dense, almost spherical, and poorly deformable. Previous reports suggest that loss of membrane and oxidation of fetal Hb might account for the generation of the dense, rigid RBC in neonates and the shortened life-span of neonatal RBC. To test whether the dense RBC population is particularly fragile and which mechanical properties are responsible for the rigidity of these cells, the following measurements were made for the top (least dense) and bottom (most dense) 3% fractions of density-separated neonatal and adult RBC: cellular deformability (rheoscope); RBC geometry (micropipette system); elasticity, fragility, and viscosity of RBC membrane (micropipette system); Hb solution viscosity (cone-plate viscometer); and selected biochemical parameters. Fetal Hb of neonatal RBC decreased with increasing cell density. When the bottom fractions were compared with the top fractions, neonatal RBC showed a greater reduction in glutamic oxalacetic transaminase activity (72% *versus* 53%), potassium (39% *versus* 19%), volume (32% *versus* 19%), and surface area (42% *versus* 21%), and a greater rise in density (3.5% *versus* 1.9%) and mean corpuscular Hb concentration (42% *versus* 23%) than adult RBC. Cellular deformability in the rheoscope (shear stress 5 Pa) decreased by 24% in adults and by 41% in neonates. Membrane extensional and bending elastic moduli (*i.e.* membrane deformability) and membrane fragility of neonatal and adult RBC did not significantly change with increasing cell density. However, the membrane surface viscosity increased by 175% in the neonatal RBC and by 76% in the adult RBC when the bottom fractions were compared with the top fractions. Hb solution viscosity increased by 256% in neonatal and by 110% in adult RBC. But at a given Hb concentration, Hb solution viscosity was similar in neonates and adults. The results indicate that the densest neonatal RBC are not particularly fragile and that the poor deformability of these cells results from loss of membrane surface area and rise of membrane and Hb viscosity. (*Pediatr Res* 34: 688–693, 1993)

Abbreviations

GOT, glutamic oxalacetic transaminase
HbF, fetal Hb
MCHC, mean corpuscular Hb concentration
MCV, mean corpuscular volume
RBC, red blood cell

Received August 24, 1992; accepted April 6, 1993.
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Supported by a research grant (Li 291/4-3) from the Deutsche Forschungsgemeinschaft and by the National Institutes of Health (Grants HL15922 and HL41341).

Separation of normal human RBC according to their density results in the isolation of distinct RBC subpopulations. Increase in density is associated with loss of water, volume, and surface area, decrease in several enzyme activities, and increase in MCHC (1, 2). RBC deformability in adults decreases progressively with increasing cell density, due to decreasing surface area (*i.e.* increasing sphericity) and increasing membrane and Hb viscosity (3–6).

Neonatal RBC show a markedly broader density distribution with a higher proportion of very light and extremely dense cells compared with adult RBC (7–9). Gahr *et al.* (7) observed that 15% of neonatal RBC have a density of 1.11 g/mL or more, whereas only 5% of adult RBC show such a high density. The densest neonatal RBC show a greater increase in MCHC and a more pronounced decrease in enzyme activities, volume, surface area, and deformability than the densest adult RBC (1, 8–10). A recent paper suggests that increased oxidation of HbF may contribute to the creation of the subpopulation of extremely dense RBC in neonates (11).

Dense RBC bind more IgG (9) and are quickly removed (12). This led to the assumption that RBC become more dense as they age and that density separation techniques can be used to obtain RBC of different ages (2). However, recent studies on *in vivo* aged RBC suggest that the densest RBC are not much older than unfractionated RBC and that dense RBC are formed at an earlier stage (13). Because dense RBC are quickly removed (12), increased generation of dense RBC may explain why the life-span of neonatal RBC ranges from 45 to 70 d (14), whereas in adults most RBC show a fairly constant survival time of 120 d (2).

In the present investigation, we have studied several mechanical properties, surface area, volume, and deformability of density-separated neonatal and adult RBC to assess the following questions: *1*) Do the dense neonatal RBC represent a cell population with increased mechanical fragility of the membrane [similar to congenital spherocytosis (15)]? *2*) Which of the determinants of RBC deformability (excess surface area, shape, membrane elasticity, membrane and internal viscosity) are responsible for the marked decline in the deformability of the dense neonatal RBC subpopulation? *3*) Does the high HbF content of dense fetal RBC (7) contribute to the decrease in cellular deformability?

MATERIALS AND METHODS

Blood samples and RBC preparation. Placental blood samples from 15 healthy, vaginally born newborn infants with gestational

ages of 39 to 40 wk and birth weights of 3250 to 3630 g were studied with the approval of the Department of Pediatrics Human Subjects Research Committee. The umbilical artery pH was above 7.25, the 1-min Apgar score was 9 and the 2- and 5-min Apgar scores were 10 in all cases. Blood (20 mL) was collected from the placenta into EDTA (approximately 1 mg/mL) immediately after cord clamping before delivery of the placenta. Adult blood samples (20 mL) were collected from 15 healthy male laboratory personnel (aged 21 to 35 y, nonsmokers, not obese) by venipuncture into EDTA. Ten neonatal and ten adult blood samples were collected for the micropipette studies; five neonatal and five adult blood samples were used for the measurements of RBC density and Hb solution viscosity. Blood samples were stored on ice during the transport to the laboratory and analyzed within 4 h after collection.

Part of each neonatal and adult blood sample was used for density separation as described in previous reports (4, 10). Whole blood was placed into 4-mm internal diameter by 47-mm long plastic tubes and centrifuged for 15 min at 12 000 × *g*. After centrifugation, the length of the red cell column was accurately measured and each tube was then cut at the border between the buffy coat and the RBC; the top 3% of the cells was then carefully removed. The tube was cut again very close to the lower end and the bottom 3% of the cells was removed. Top and bottom fractions thus contained 3% of the least and most dense cells, respectively. This method for RBC density separation does not use gradient media and thus does not separate RBC fractions with defined absolute densities. However, an important advantage of this method is that small quantities of top and bottom fractions are rapidly available: Neonatal RBC have a tendency to assume an echinocytic shape if not studied promptly (16), and the rheologic and mechanical properties of RBC are very sensitive to shape changes (17).

Micropipette system. Details of the microscope-video system, the micropipette system, the production and handling of micropipettes (18, 19), and the RBC preparation (4, 20) have been described previously. RBC were diluted at a hematocrit of about 0.001 L/L in PBS containing 1 g/L human serum albumin. Note that one neonatal and one adult blood sample were studied on the same day, using the same micropipette; 30 RBC were tested in each sample for each micropipette method described below. Less than 5% of RBC in each sample showed microscopically visible echinocytic alterations. Echinocytes were excluded from the study because their mechanical properties may be altered (17).

RBC membrane elastic moduli, fragility, and viscosity. Pipettes with internal diameters of 1.4 to 1.6 μm were used for studies of RBC recovery time from extensional deformation (21), membrane extensional and bending elastic moduli (6), and mechanical membrane fragility (19); each of these four measurements were carried out on the same RBC (19, 20).

The time constant for extensional shape recovery was studied by the method of Hochmuth *et al.* (21) and calculated as described by Nash and Meiselman (5). The pipette was used to extend (overall extension ratio of approximately 1.6) and quickly release point-attached RBC. From video recordings of the recovery process, the cell length-width ratio was measured every [1/50] s for 20 fields after releasing the cell. The extensional and bending elastic modulus and the mechanical fragility were then studied after the extended cell had recovered its initial shape (19, 20). A small membrane tongue was aspirated into the pipette from a flat portion of the cell surface (6, 22). Tongue length (L_T) was measured from video recordings at a series of increasing aspiration pressures (P). Initially, a smooth membrane tongue forms in the pipette. Then the membrane begins to fold and finally the membrane ruptures. These sequential events were videorecorded and used to determine the extensional elastic modulus, the bending elastic modulus, the yield strain, and the yield tensile stress of the RBC membrane. The extensional (shear) elastic modulus (μ_e) was calculated from a linear relationship that exists between L_T/R_p (R_p = pipette radius) and $P \cdot R_p$ over a L_T/R_p range approximately between 1.5 and 3 (6):

$$L_T/R_p \propto (1/\mu_e) \cdot P \cdot R_p$$

For a given pipette radius, μ_e defines the pressure required to produce a defined membrane tongue length. In other words, μ_e determines RBC membrane deformation in response to a given force. A small μ_e indicates little resistance to deformation and high membrane flexibility. The membrane viscosity was calculated as product of the time constant for extensional shape recovery and the extensional elastic modulus (6). Membrane bending elastic modulus (*i.e.* resistance to membrane bending and folding) was derived from the pressure at which the membrane began to fold during aspiration of the membrane tongue into the micropipette (6, 20).

The strain (ϕ) in the cylindrical section of the aspirated membrane tongue is (19, 22):

$$\phi = (1/4)\,[(A_p/R_p^2) + \ln(1 + A_p/R_p^2)]$$

$$A_p = (A_{cap} + A_{cyl} - \pi R_p^2)/\pi$$

where A_{cap} is the surface area of the cap of the aspirated membrane tongue and A_{cyl} is the surface area of the cylinder portion. The strain ϕ in the membrane reflects the extent of membrane deformation (*i.e.* the membrane tongue length in the pipette). The yield strain of the membrane defines the maximum deformation that the membrane can reach before it yields and ruptures. $\phi \times \mu_e$ is the tensile stress. Tensile strength at the yield point of the membrane is the yield tensile stress.

RBC geometry. Pipettes with internal diameters of 1.8 to 2.0 μm were used for the determination of RBC surface area and volume (23); the resting diameters of RBC were measured before micropipette aspiration. Other RBC geometrical characteristics, *i.e.* mean thickness, surface area index, swelling index, and minimum cylindrical diameter, were calculated based upon cell diameter, surface area, and volume. Note that both the surface area index and the swelling index have a limiting value of 1.0 for a spherical shape. A surface area of 1.40 indicates an excess surface area beyond that required to enclose the cellular volume of 40%. A swelling index of 1.65 indicates that the volume of a RBC can swell by 65% (swelling capacity) before it reaches spherical shape and hemolyzes. The minimum cylindrical diameter is the width of an RBC with cylindrical shape and defines the narrowest cylindrical tube through which an RBC can pass.

Hb solution viscosity. Hb solutions were prepared from unfractionated RBC and from top and bottom fractions of five neonatal and five adult blood samples according to the method described by Cokelet and Meiselman (24). The Hb concentrations of the solutions from top and bottom RBC were adjusted to the mean intracellular Hb concentrations determined in the first part of the study for the corresponding cell types (Table 1). The concentrations of Hb solutions from unfractionated neonatal and adult RBC were also adjusted at the mean values determined in the first study for unfractionated, top, and bottom cells. Hb solution viscosities were measured at 37°C using a Wells-Brookfield cone-plate viscometer (Brookfield Engineering Labs., Stoughton, MA).

RBC deformability. Deformability of single RBC was assessed using a counter-rotating, cone-plate rheoscope (25) (Effenberger, Munich, Germany) that was mounted on an inverted microscope (Leitz Diavert, Wetzlar, Germany). Details of the method have been described elsewhere (26). For the deformability measurements, RBC were diluted at a hematocrit of about 0.01 L/L in PBS containing 200 g/L dextran T-70 (Pharmacia, Uppsala, Sweden). The osmolality of these dextran solutions was 295 ± 5 mosmol/kg and the viscosity was 21 mPa·s at 25°C. Shear stresses of 0.5, 5, and 50 Pa were applied, and the elongation of single RBC was evaluated on photomicrographs. Length (L) and width (W) of 40 RBC were measured in each sample at each

Table 1. *General properties of unfractionated, top, and bottom RBC**

		Top	Unfractionated	Bottom
Mean density (g/mL)	N	1.079 ± 0.005†‡	1.099 ± 0.005‡	1.119 ± 0.006†
	A	1.089 ± 0.004‡	1.101 ± 0.003‡	1.111 ± 0.003
MCV (fL)	N	129.7 ± 12.3†‡	105.7 ± 5.1†‡	88.1 ± 4.7†
	A	96.8 ± 5.5‡	89.5 ± 3.9‡	79.7 ± 3.5
MCH (fmol)	N	2.19 ± 0.15†	2.17 ± 0.11†	2.14 ± 0.21†
	A	1.83 ± 0.12	1.85 ± 0.09	1.81 ± 0.10
MCHC (mmol/L)	N	16.9 ± 1.6†‡	20.4 ± 0.9‡	24.0 ± 1.4†
	A	18.6 ± 1.1‡	20.6 ± 0.8‡	22.9 ± 1.1
GOT (IU/mmol Hb)	N	332 ± 87†‡	137 ± 47†‡	92 ± 40†
	A	130 ± 40‡	76 ± 24	61 ± 23
K^+ (mmol/mmol Hb)	N	1905 ± 264†	1758 ± 137†‡	1171 ± 93†
	A	1676 ± 121	1575 ± 95‡	1357 ± 103
HbF (%)	N	36.4 ± 8.7†‡	65.7 ± 7.8†	73.6 ± 9.5†
	A	0.6 ± 0.3	0.7 ± 0.3	0.8 ± 0.4
Reticulocytes (%)	N	25.3 ± 9.6†‡	5.0 ± 1.8†‡	0.4 ± 0.2
	A	10.9 ± 2.7‡	1.2 ± 0.4‡	0.2 ± 0.1

* Data represent mean ± 1 SD of the sample means. MCH, mean corpuscular Hb; A, adults; N, neonates. MCV was obtained via the Contraves system. Mean RBC density was determined in five neonatal and five adult blood samples, whereas the other parameters were studied in 10 neonatal and 10 adult blood samples.

† Neonatal and adult RBC (same fraction) significantly different; $p < 0.05$ (unpaired test).

‡ Top or bottom RBC significantly different from unfractionated RBC; $p < 0.05$ (paired-difference test).

shear stress, with RBC elongation (E) expressed as E = (L − W)/(L + W). E increases with increasing cellular deformation.

Miscellaneous techniques. RBC count, MCV, and Hb concentration were measured using a cell counter (Contraves, Zürich, Switzerland). Reticulocytes were counted after the staining of a blood smear by brilliant cresyl blue. HbF was quantified by the alkali denaturation test (27). GOT was measured with a test kit (Boehringer, Mannheim, Germany) (7). Intraerythrocytic potassium concentration was determined by flame photometry (28). Density distribution of fractionated and unfractionated RBC was studied by the phthalate-oil method (29) as reported elsewhere (13).

Statistical analyses. Arithmetic means were calculated for 30 (micropipette studies) or 40 (rheoscope) RBC tested in each sample of unfractionated, top, and bottom fractions. These sample means were used to calculate the means and SD for each parameter. One-way analysis of variance was used to test for overall differences between unfractionated, top, and bottom RBC fractions in neonates and adults. If the overall F ratio was found to be significant ($p < 0.05$), Tukey's honestly significant-difference test (30) was used to analyze *1*) the differences of corresponding RBC fractions between neonates and adult (unpaired comparisons), and *2*) the differences among various RBC fractions separately for neonates and adults (paired comparisons).

RESULTS

Unfractionated RBC. The hematologic, geometric, and mechanical properties of unfractionated RBC are shown in Tables 1–3. MCV obtained via Contraves cell counter (Table 1) and volume determined by means of micropipette system (Table 2) were not significantly different. MCV, mean corpuscular Hb, HbF, GOT activity, and potassium concentration of neonatal RBC were markedly higher than those of adult RBC (Table 1). Figure 1 shows that the density of neonatal RBC ranged from 1.074 to 1.122 g/mL, whereas the density of adult RBC ranged from 1.082 to 1.118 g/mL. Compared with adult RBC, the volume of neonatal RBC was 18% larger, their surface area was 12% greater, and their diameter was 11% wider (Table 2). The surface area to volume ratio of the neonatal RBC was significantly decreased and their minimum cylindrical diameter was increased compared with adult cells. There were no significant differences between neonatal and adult RBC for mean thickness, surface area index, and swelling index.

Cellular deformability as measured in the rheoscope did not show significant differences between the neonates and adults at any of the applied shear stresses (Table 3). Both the extensional and the bending elastic moduli of the neonatal RBC were 12% smaller, whereas the time constant for recovery from extensional deformation was 25% larger than that for adult cells. Membrane viscosity and the yield strain were similar in neonates and adults. The yield tensile stress was 21% lower in neonates than in adults.

Fractionated RBC. GOT activity decreased markedly from the top fraction to the unfractionated cells, whereas the potassium concentration decreased mainly from the unfractionated to the bottom cells (Table 1). HbF in the neonatal bottom RBC was two times higher than in the top fraction, but similar in the unfractionated and bottom RBC.

When the bottom fractions were compared with the top fractions, neonatal RBC showed a greater rise in density (3.5% *versus* 1.9%) and MCHC (42% *versus* 23%) than adult RBC (Table 1). Moreover, neonatal RBC lost about twice as much surface area (−42% *versus* −21%) and volume (−32% *versus* −19%) as adult RBC (Table 2). The excess surface area decreased from 49 ± 7% to 12 ± 4% for the neonatal RBC and from 46 ± 5% to 33 ± 4% for adult RBC when the bottom fractions were compared with the top fractions (Table 2). The swelling capacity of RBC decreased from 82 ± 8% to 19 ± 5% in the neonates and from 75 ± 6% to 53 ± 5% in the adults. Thus, the dense neonatal RBC had much less excess surface area (12% *versus* 33%) and swelling capacity (19% *versus* 53%) than the dense adult RBC.

For both neonatal and adult RBC, the membrane extensional and bending elastic moduli, the yield strain, and tensile stress did not significantly change with increasing cell density (Table 3). The time constant for recovery from extensional deformation of neonatal and adult RBC increased markedly with increasing density, with the increase being more pronounced for neonatal RBC than for adult cells (+140% *versus* +60%). Note that the most dense neonatal RBC needed 50% more time for recovery from extensional deformation than the most dense adult RBC (Table 3).

The calculated membrane viscosity was also increased more for the dense neonatal RBC than for the dense adult RBC compared with the corresponding least dense (top) cell fractions (+175% *versus* +76%). Figure 2 shows the Hb solution viscosities as functions of MCHC; it is clear that a single curve describes the data for all adult and neonatal samples. Table 4 presents the viscosities of various Hb solutions, where at any given Hb concentration, the viscosity was similar for solutions prepared from neonatal and adult RBC or from fractionated and unfrac-

Table 2. *Geometric data for unfractionated, top, and bottom RBC**

		Top	Unfractionated	Bottom
Volume (fL)	N	130.8 ± 13.1†‡	107.3 ± 5.6†‡	89.4 ± 5.9†
	A	99.2 ± 6.3‡	90.5 ± 4.4‡	80.3 ± 4.0
Surface area (μm^2)	N	186.1 ± 10.6†‡	153.5 ± 7.0†‡	108.5 ± 8.2†
	A	150.6 ± 8.4‡	137.1 ± 6.7‡	118.6 ± 6.2
Surface area/volume	N	1.43 ± 0.05†	1.43 ± 0.04†‡	1.21 ± 0.05†
	A	1.51 ± 0.03	1.51 ± 0.04	1.49 ± 0.04
Diameter (μm)	N	9.8 ± 0.4†‡	8.8 ± 0.4‡	6.9 ± 0.5
	A	8.6 ± 0.5‡	7.9 ± 0.4	7.5 ± 0.4
Mean thickness (μm)	N	1.70 ± 0.11	1.76 ± 0.10‡	2.39 ± 0.13†
	A	1.71 ± 0.11‡	1.84 ± 0.13	1.83 ± 0.14
Surface area index	N	1.49 ± 0.07‡	1.40 ± 0.05‡	1.12 ± 0.04†
	A	1.46 ± 0.05‡	1.41 ± 0.04‡	1.33 ± 0.04
Swelling index	N	1.82 ± 0.08‡	1.67 ± 0.06‡	1.19 ± 0.05†
	A	1.75 ± 0.06‡	1.66 ± 0.04‡	1.53 ± 0.05
Minimum cylindrical diameter (μm)	N	2.93 ± 0.12	3.00 ± 0.14†‡	3.83 ± 0.32†
	A	2.80 ± 0.12	2.83 ± 0.14	2.89 ± 0.13

* Data represent mean ± 1 SD of the sample means. A, adults; N, neonates. Volume and surface area were obtained via a micropipette system. Each parameter was studied in 10 neonatal and 10 adult blood samples.

† Neonatal and adult RBC (same fraction) significantly different; $p < 0.05$ (unpaired test).

‡ Top or bottom RBC significantly different from unfractionated RBC; $p < 0.05$ (paired-difference test).

Table 3. *Mechanical properties of unseparated, top, and bottom RBC**

		Top	Unfractionated	Bottom
Extensional elastic modulus (μ_e; 10^{-6} N/m)	N	5.2 ± 0.6†	5.4 ± 0.7†	5.8 ± 0.7
	A	5.9 ± 0.5	6.2 ± 0.6	6.5 ± 0.8
Time constant (t_e) for extensional recovery (s)	N	0.10 ± 0.02‡	0.15 ± 0.02†‡	0.24 ± 0.03†
	A	0.10 ± 0.02‡	0.12 ± 0.02‡	0.16 ± 0.03
Membrane viscosity ($\mu_e \cdot t_e$; 10^{-6} N·s/m)	N	0.53 ± 0.10‡	0.76 ± 0.15‡	1.46 ± 0.24†
	A	0.59 ± 0.11‡	0.78 ± 0.13‡	1.04 ± 0.18
Bending elastic modulus (10^{-18} N·m)	N	14.3 ± 2.6†	15.5 ± 2.7†	16.0 ± 2.3†
	A	16.2 ± 2.5	17.1 ± 2.2	17.9 ± 3.4
Yield strain	N	6.0 ± 0.6	6.4 ± 0.9	6.6 ± 0.9
	A	6.5 ± 0.9	6.7 ± 1.0	7.0 ± 1.4
Yield tensile stress (10^{-6} N/m)	N	31.5 ± 4.3†	33.9 ± 4.0†	37.3 ± 5.2†
	A	39.8 ± 5.7	43.4 ± 6.1	45.6 ± 6.8
RBC deformation at 5 Pa (rheoscope)	N	0.51 ± 0.03†‡	0.41 ± 0.03‡	0.32 ± 0.04†
	A	0.47 ± 0.03‡	0.42 ± 0.03‡	0.36 ± 0.04

* Data represent mean ± 1 SD of the sample means. A, adults; N, neonates. Each parameter was studied in 10 neonatal and 10 adult blood samples.

† Neonatal and adult RBC (same fraction) significantly different; $p < 0.05$ (unpaired test).

‡ Top or bottom RBC significantly different from unfractionated RBC; $p < 0.05$ (paired-difference test).

tionated RBC. However, for solutions prepared at Hb concentrations appropriate for the fractionated cells, significant differences were observed (adult top 31% greater than neonatal top, adult bottom 23% less than neonatal bottom).

The most dense RBC showed significantly decreased deformability in the rheoscope compared with the least dense RBC (Table 3). The least dense neonatal RBC had significantly higher deformability than the least dense adult cells, whereas the most dense neonatal RBC were less deformable than the most dense adult RBC. Figure 3 indicates that RBC deformation, at constant shear stress, decreased with increasing MCHC for both cell types.

DISCUSSION

In agreement with previous studies (7–9, 29), we observed a wider density range for neonatal RBC (Fig. 1). The greater increase in density of neonatal RBC from the top to the bottom fraction was associated with greater rise in MCHC and a more pronounced decrease in MCV and surface area (Table 2). Matovcik *et al.* (8) report smaller volumes and surface areas for the top fraction of neonatal RBC, probably because their top fraction comprised 15% of the RBC, whereas we separated only 3% of the top cells.

The production of HbF decreases by 3% per week from 26 to 43 wk of gestation (31). This implies that the HbF content of the youngest neonatal RBC should be 30% lower than the HbF of the oldest cells, because the oldest RBC in full-term neonates are about 10 wks old (14). This corresponds to our finding of a 37% difference in HbF between top and bottom RBC in neonates (Table 1). In particular, we found considerably less HbF in the top fraction of neonatal RBC compared with unfractionated cells (Table 1). This indicates that the least dense neonatal RBC are younger than the average unfractionated neonatal RBC. The average unfractionated RBC survives about 4 wk in the circulation (14). Thus, HbF in the oldest neonatal RBC should be about 12% higher than in the unfractionated RBC. The most dense neonatal RBC showed 8% more HbF than unfractionated neonatal RBC (Table 1). Because the difference was not statistically significant, we cannot conclude from this result that in neonates the most dense RBC are older than unfractionated RBC. A recent study on *in vivo* aged RBC in children with transient erythroblastopenia indicated that the most dense RBC are not much older than unfractionated RBC (13).

The exact mechanism for the loss of membrane surface area from dense cells is unknown. Four possible mechanisms may be discussed: *1*) Membrane may be lost by endocytosis (8). *2*) RBC membrane fragments may be removed by immunologic mechanisms (9). *3*) HbF and other proteins in neonatal RBC are highly

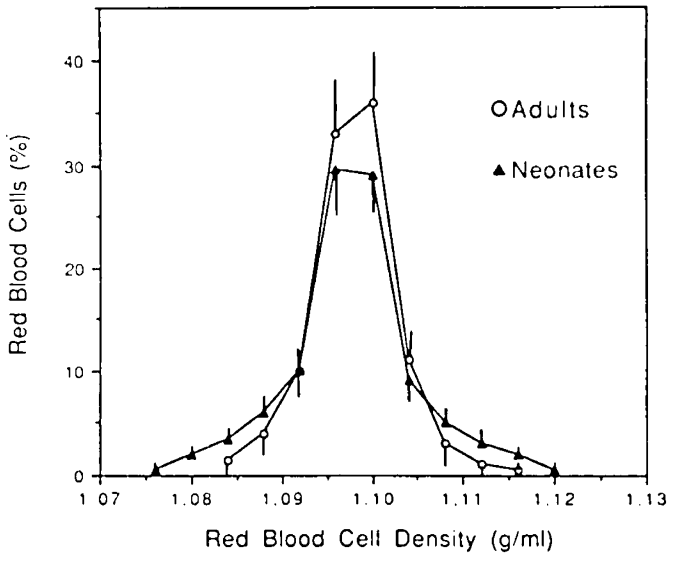

Fig. 1. Density distribution of unfractionated RBC in full-term newborn infants (▲) and adults (O). Note that the distribution in neonates was broader than in adults.

susceptible to oxidative damage (11). *4*) The continuous mechanical stress acting on the membrane of circulating RBC may result in fragmentation of the RBC membrane (15). Mechanical fragmentation of RBC membrane is principally a result of extreme elastic membrane deformation. RBC have to be extremely flexible to pass through narrow capillaries and splenic slits, whereas the RBC membrane must withstand the continuous strain resulting from frequent deformations. In general, extremely flexible (*i.e.* highly elastic) membranes are less resistant to mechanical fragmentation (*e.g.* spherocytosis) than membranes with normal elasticity (15).

The smaller elastic moduli and the reduced critical tensile stress for membrane fragmentation of neonatal RBC (Table 3) indicate that the membrane of neonatal RBC is more deformable and more fragile than the membrane of adult RBC. Compared with adult RBC, the membrane of neonatal RBC ruptured at smaller pressure (*i.e.* smaller tensile stress) but at similar extent of membrane deformation (*i.e.* similar strain). A recent study on RBC membrane fragility using a flow channel showed that neonatal RBC lose more membrane per unit time (19). Thus, the membrane of neonatal RBC is more fragile due to increased membrane deformability and faster membrane loss. Because membrane elasticity and fragility did not significantly change with increasing cell density (Table 3), the dense neonatal RBC do not represent a cell population with increased mechanical fragility of the membrane. Moreover, our results suggest that increased binding of oxidized Hb to the membrane of the densest neonatal RBC (11) does not alter their membrane elasticity and fragility.

The biochemical basis for the different membrane mechanical properties of neonatal RBC is unknown. Neonatal and adult RBC show no qualitative or gross quantitative differences in the pattern of the membrane proteins (9), but neonatal RBC contain a greater proportion of unsaturated fatty acids (16), and membrane lipids of neonatal RBC are more susceptible to oxidative damage (32).

Because Hb solution viscosities in unfractionated and fractionated RBC were similar at given Hb concentrations (Table 4), we conclude that the increase in Hb viscosity in the densest RBC was due solely to the elevated Hb concentration and not to altered Hb properties (*e.g.* oxidized Hb) (11). The increase in MCHC of dense RBC is associated with an increase in membrane-bound Hb (9, 11). Increased membrane-bound Hb results in increased membrane shear viscosity but does not alter membrane elasticity (33). Thus, the increased membrane viscosity in the most dense neonatal RBC (Table 3) is probably a result of increased membrane-associated Hb (9, 11). It is unclear whether increased membrane-attached oxidized Hb in dense neonatal RBC (11) contributes to the high membrane viscosity.

RBC deformability depends on cell geometry (*i.e.* excess surface area and shape), membrane deformability (*i.e.* extensional and bending elastic modulus), and viscous properties (*i.e.* membrane and Hb viscosity) (17): *1*) The excess surface area determines the maximum extent of whole cell deformation; *2*) membrane elasticities determine the forces required for a given extent of membrane deformation; and *3*) membrane viscosity limits the rate of membrane deformation, whereas the Hb viscosity determines the rate of cell bending and folding (6). Deformation of neonatal RBC decreased markedly more compared with adult

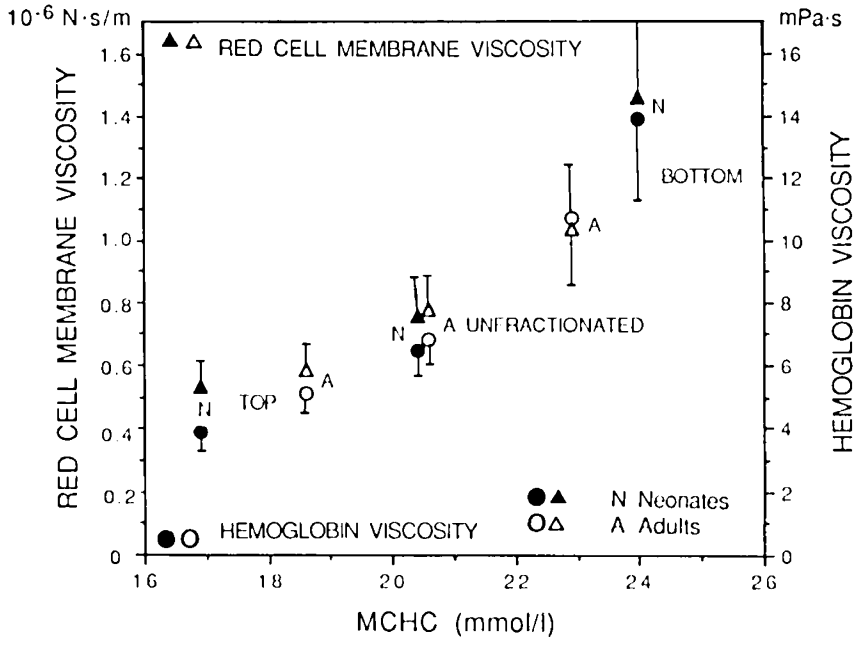

Fig. 2. Membrane viscosity (*triangles*) and Hb viscosity (*circles*) of top (light), bottom (dense), and unfractionated neonatal (*closed symbols*) and adult (*open symbols*) RBC plotted as a function of MCHC. Data represent mean ± 1 SD for 10 neonatal and 10 adult samples.

Table 4. *Viscosity of Hb solutions prepared from neonatal and adult RBC**

Hb concentration (mmol/L)	Hb solution viscosity (mPa·s)		
	Unfractionated red cells		Top or bottom red cells
	N	A	
18.8	3.5 ± 0.5	3.7 ± 0.4	3.9 ± 0.6 (N,T)†
18.6	4.6 ± 0.7	4.8 ± 0.6	5.1 ± 0.7 (A,T)
20.5	6.5 ± 0.9	6.8 ± 0.8	
23.0	9.8 ± 1.6	10.3 ± 2.1	10.7 ± 1.9 (A,B)
24.2	12.4 ± 2.0	13.0 ± 2.9	13.9 ± 2.8 (N,B)†

* Values are mean ± 1 SD; A, adults; N, neonates; B, bottom red cells; T, top red cells. Hb concentrations were adjusted to the mean MCHC values found in the first part of the study (Table 1). The studies were performed in five neonatal and five adult blood samples.

† Neonatal and adult RBC (same fraction) significantly different $p < 0.05$ (unpaired test).

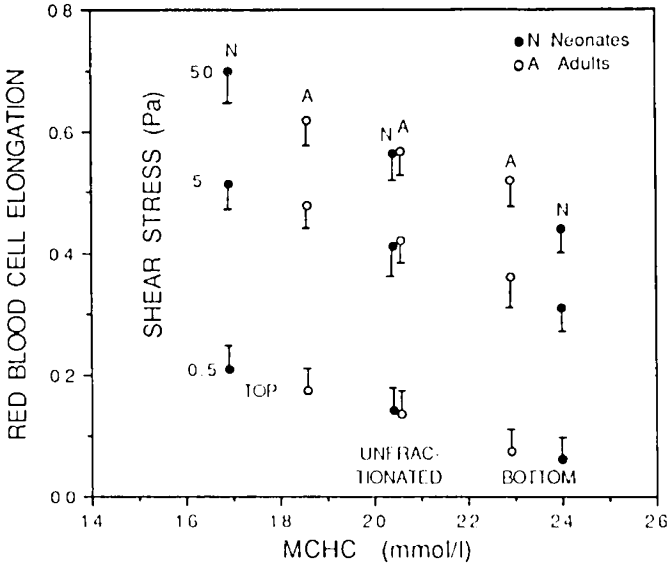

Fig. 3. Elongation (measured in the rheoscope) of top (light), bottom (dense), and unfractionated neonatal (●) and adult (○) RBC plotted as functions of MCHC and shear stress. Data represent mean ± 1 SD for 10 neonatal and 10 adult samples.

RBC when the bottom fractions were compared with the top fractions (Table 3). Our results indicate that this can be explained by a more pronounced loss of surface area and a greater rise in membrane and Hb viscosity in the densest neonatal RBC.

We conclude that neonatal RBC are more fragile than adult RBC, but that the densest neonatal RBC are neither more fragile nor much older than the average circulating RBC in the neonate. This suggests that the formation of dense RBC cannot be explained by peculiar mechanical properties. The densest neonatal RBC are extremely rigid due to decreased excess surface area and increased membrane and internal viscosity. Because dense RBC are quickly removed (12), enhanced formation of dense RBC may contribute to the markedly shorter life-span of neonatal RBC (14).

Acknowledgments. The authors thank Liselotte B. A. Krüger for secretarial assistance and Vivian M. Vargas, M.S.B.A., and John C. Lockhart, Ph.D., for their help in preparing the manuscript.

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