Rheologic Properties of Senescent Erythrocytes: Loss of Surface Area and Volume With Red Blood Cell Age

By Richard E. Waugh, Mohandas Narla, Carl W. Jackson, Thomas J. Mueller, Takashige Suzuki, and George L. Dale

The rheologic properties of senescent erythrocytes have been examined using two models of red blood cell (RBC) aging. In the rabbit, aged erythrocytes were isolated after biotinylation, in vivo aging, and subsequent recovery on an avidin support. Aged RBCs from the mouse were obtained using the Ganzoni hypertransfusion model that suppresses erythropoiesis for prolonged periods of time allowing preexisting cells to age in vivo. In both cases, the aged erythrocytes were found by ektacytometry to have decreased deformability due to diminished surface area and cellular dehydration. The aged rabbit erythrocytes were further characterized by micropipette methods that documented an average surface area decrease of 10.5% and a volume decrease of 8.4% for the cells that were 50 days old. Because both the surface area and volume decreased with cell age, there was little change in surface-to-volume ratio (sphericity) during aging. The aged cells were found to have normal membrane elasticity. In addition, human RBCs were fractionated over Stratrac density gradients and the most dense cells were found to have rheologic properties similar to those reported for the aged RBCs from rabbits and mice, although the absolute magnitude of the changes in surface area and volume were considerably greater for the human cells. Thus, stringent density fractionation protocols that result in isolation of the most dense 5% of cells can provide a population of human cells with rheologic properties similar to senescent cells obtained in other species. The data indicate that progressive loss of cell area and cell dehydration are characteristic features of cell aging.

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MATERIALS AND METHODS

Isolation of Aged Erythrocytes

The average lifespan of rabbit and mouse erythrocytes is 60 days. Aged erythrocytes from rabbits were isolated with a recently reported method that involves the biotinylation of erythrocytes with N-hydroxysuccinimido biotin.21 Specifically, 2.5 kg rabbits were injected on 3 consecutive days with 7.5 mg/kg phenylhydrazine to produce a reticulocytosis; control experiments have shown that 70% to 85% of all preexisting cells are destroyed by this procedure (data not shown). Ten days later, the rabbit RBCs are biotinylated in vitro by reaction with N-hydroxysuccinimido biotin as previously described.22 These biotinylated cells have been shown to have a normal in vivo survival.23 After reinfusion, the biotinylated cells are allowed to age in vivo for various periods of time. The animal is then bled again and the biotinylated RBCs are recovered by binding to avidin-coated, plastic Petri dishes as described.22 The recovered cells are removed from the Petri dishes.

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1351
by collagenase digestion of the gelatin anchor\textsuperscript{23} used to retain the avidin on the plate surface. One advantage of this technique is that when an age-synchronized cohort of cells is initially used for the biotinylation, any desired age of erythrocyte can be obtained, thereby allowing a temporal dissection of the aging process relative to the parameter being examined.\textsuperscript{24,25} As mentioned above, the phenylhydrazine treatment does not quantitatively remove existing cells so that the purity of the isolated RBC preparations is not as great for an early isolation (ie, day 8) as it is for the more aged preparations where any cells outside the initial age-window would have been cleared by the natural senescence process. Isolated RBC populations, as well as whole blood samples, were resuspended in autologous plasma at 25% hematocrit and shipped on wet ice via overnight carrier to the laboratories of N.M. and R.E.W. for rheologic measurements.

Aged erythrocytes from the mouse were isolated with the Ganzoni hypertransfusion procedure.\textsuperscript{23,24} With this technique, a large number of starting mice are split into two equal groups, and one group is terminally bled to allow the other group of animals to be hypertransfused. Two weeks later, one half of the surviving animals are terminally bled to hypertransfuse the remaining mice; this procedure is repeated every 2 weeks for approximately 60 days. The model is based on the observation that hypertransfused animals will not synthesize new erythrocytes, and, therefore, the RBC population of the few surviving animals will have a continuously increasing mean age over the 60-day experiment.

There are important differences between the biotinylation and hypertransfusion models. When the hypertransfusion procedure has been proceeding for 30 days, the surviving RBCs represent a range of ages from 30 to 60 days old. Only at the end of the hypertransfusion experiment does the age-window of the remaining cells become relatively narrow. In contrast, the age-window in the biotinylation system is determined by the starting population, which is a phenylhydrazine-produced, young cohort of cells. Therefore, the age-window for these cells is approximately 10 days wide and will remain constant during the experiment. However, the use of phenylhydrazine in the biotinylation model must be considered for the potential of introducing artifacts based on the use of this oxidant challenge in the animal.

**Density Separation of Human RBCs**

Human RBCs were fractionated using discontinuous eight-step stractan density gradients consisting of 1 mL fractions spanning a density range of 1.084 to 1.120 g/mL in equal increments.\textsuperscript{27} The RBCs from the most dense fraction had a mean cellular hemoglobin concentration (MCHC) greater than 37 g/dL and constituted \( \sim 0.8\% \) of the total cell population.

**Ektacytometry Measurements**

Deformability of intact RBCs was measured using osmotic gradient ektacytometry, an assay in which whole-cell deformability is measured as a continuous function of suspending medium osmolality.\textsuperscript{4} For these measurements, we prepared gradients from two solutions of 4% polyvinyl-pyrrolidone (PVP; average molecular weight 360 Kd and viscosity of 22 cp) in phosphate-buffered saline (PBS), one adjusted to 50 mOsm/kg and the other to 900 mOsm/kg. The gradients were mixed in the first stage of the three-stage mixing chamber of a Beckman gradient former (Beckman Instrument Co, Palo Alto, CA). Packed RBCs (70% to 80% hematocrit) were pumped into the second stage of the chamber by a Harvard infusion pump (Model No. 906; Harvard Apparatus, South Natick, MA) and mixed with the gradient to a final hematocrit of 0.2%. Thorough mixing was ensured by passage of the cell suspension through the third stage of the mixing chamber. The suspension was then pumped through a Wescan conductivity meter (Wescan Instruments, Santa Clara, CA) to continuously monitor its conductivity, and finally into the ektacytometer for measurement of cellular deformability, at a constant shear stress of 170 dyne/cm\(^2\). The osmolality at which the deformability index (DI) reaches a minimum in the hypotonic region of the gradient has been shown to be the same as the osmolality at which 50% of the cells will hemolyze in a standard osmotic fragility test.\textsuperscript{5} This point is thus an index of the average surface area-to-volume ratio of the population of cells studied. Cells attain their maximally deformed state at or near the physiologically relevant osmolality of 290 mOsm. In the presence of normal membrane deformability, this maximum value of DI has previously been shown to be related to the membrane surface area.\textsuperscript{7} The hypertonic region of the curves provides information on the state of cell hydration.

Variations in cell hemoglobin concentration distributions in aging RBC populations was quantitated using the Technicon H-1 hematology analyzer (Technicon Instruments Corporation, Tarrytown, NY).\textsuperscript{11}

**Micropipette Measurements**

The surface areas and volumes of rabbit cells were measured on six different occasions, and four or six samples were tested on each occasion. The samples were grouped according to the age of the labeled cohort: Day\(_{22}\), Day\(_{29}\) (days 22 to 30), and Day\(_{49}\) (days 49 to 51). For each age group there was a cohort sample and a whole blood sample. Day\(_{22}\) cells were tested on three occasions, and Day\(_{29}\) cells and Day\(_{49}\) cells were tested on four occasions each. In all, over 2,000 cells were measured. The whole blood samples do not necessarily represent a "normal" cell population because the ages of the cells in the blood depend on how soon after phenylhydrazine treatment the blood is obtained. For example, for the Day\(_{22}\) sample, the control cells will represent a skewed age population because it is only 18 days after the end of phenylhydrazine treatment of the animal.\textsuperscript{20} By the time that a Day\(_{49}\) sample is drawn for the isolation of biotinylated cells, the animal will be 60 days removed from phenylhydrazine treatment and will represent a more normal distribution of cell ages. As a result, the whole blood samples from Day\(_{49}\) are most representative of normal cells.

Details of the micropipette procedures are provided elsewhere.\textsuperscript{35} Cells were suspended in PBS (128 mmol/L NaCl, 31.2 mmol/L Na phosphate, pH 7.3; 285 to 295 mOsm) plus 3.0 mg/mL bovine serum albumin at low hematocrit (<1.0%) and placed in a 1.0-mm thick, U-shaped chamber on the microscope stage. A micropipette (1.0 to 1.5 µm, inside diameter) was inserted into the chamber through the open side of the "U".

To measure the surface area and volume, cells were aspirated at a pressure of \( \sim 6,000 \) dyne/cm\(^2\) (6.0 cm water), a pressure sufficient to form the cell into a sphere plus a cylindrical projection into the pipette. The outside diameter \( R_0 \) and projection length \( L \) were measured for each cell, and the surface area \( (A) \), volume \( (V) \), sphericity \( (S) \), and minimum cylindrical diameter \( (MCD) \) were calculated according to the following relationships:

\[ A = \pi (4R_0^2 - R_0^2 + 2R_0L) \]  
(1)

\[ V = \pi (4R_0^2 - R_0^2 + 3RL)/3 \]  
(2)

\[ S = \frac{4\pi V^{1/3}}{(4/3)^{1/3}A} \]  
(3)

\[ \pi(MCD)^2 = 3A \cdot (MCD) - 12V \]  
(4)
where \( R_i \) is the inside radius of the pipette. The MCD is the diameter of the smallest tube through which the cell can pass. It was found by numerical solution of equation 4 for given values of \( A \) and \( V \) using Newton's method. Some other measures of surface to volume ratio can be calculated from the sphericity: “surface area index” \( = S^{-1} \) and “swelling index” \( = S^{-2} \). To avoid bias in cell selection, every cell in an arbitrarily chosen field of view was measured. Between 75 and 100 cells were measured for each sample. Occasionally, cells were encountered that could not be used for measurement. These included cells that creased or folded when they were aspirated into the pipette, cells that were too rigid to be deformed into the pipette, or cells that were so small that they were sucked completely into the pipette. The sum of these rejected cells amounted to less than 3% of any sample. Such cells are not included in the statistics.

To measure the deformability of the membrane, cells were aspirated near the dimple region at an initial pressure of 200 dyne/cm². The aspiration pressure was increased in increments, and the length of the projection into the pipette was measured as a function of the pressure. The modulus \( (\mu) \) was calculated according to:

\[
\mu = \left( \frac{R_i}{2.45} \right) \frac{dP}{dL}
\]

where \( dP/dL \) is the inverse of the slope of the length-pressure data pairs.

RESULTS

Ektacytometry of Erythrocytes

Erythrocytes from all three species were examined by ektacytometry to evaluate the deformability changes that may occur during the aging process. The data presented in Figs 1 through 4 address the changes that occur with increasing erythrocyte age in the rabbit and mouse and with increasing cell density in humans. Osmotic gradient deform-

![Fig. 1. Deformability of aged RBCs from the rabbit. Aged, biotinylated erythrocytes from the rabbit were isolated at the times specified. The DI of these cells was measured at a constant shear stress with variation of the osmolality of the suspending solution (mOsmol/kg). The oldest sample analyzed was 55 days; the lifespan of the rabbit RBC is approximately 60 days. Data derived from four in vivo aged blood samples is shown here. Similar patterns of deformability changes were seen in eight additional in vivo aged blood samples. The 12 deformability measurements represent data obtained from six separate animals.](image1)

![Fig. 2. Cell hemoglobin concentration for aged cell cohorts from rabbit. The difference between whole blood samples (left curves) and cohort samples increases progressively with the age of the cohort. The data for the whole blood sample shown in each panel were derived from blood samples of the animal from which the aged cohorts of RBCs were isolated. The variation in these controls represents biologic variation and differences in time since phenylhydrazine treatment. In addition to the four in vivo aged samples shown here, eight other samples were studied. The 12 samples were obtained from six different animals. The changes in cell hemoglobin concentration documented here are representative of all samples studied.](image2)

![Fig. 3. Osmotic deformability profiles of aged RBCs from the mouse. Aged RBCs were isolated from mice at various times after the initiation of hypertransfusion. The measurements here are similar to those detailed in Fig 1. The lifespan of the mouse RBC is approximately 60 days. In addition to the four in vivo aged samples shown here, similar deformability changes were seen in four blood samples of different ages obtained from four other animals.](image3)
Fractionated RBCs from four other normal donors exhibited similar deformability profiles. The density-profile of the whole blood sample from which these different density fractions were isolated is represented by the dashed line. Density-separated human RBCs. Human erythrocytes were density separated over continuous stractan density gradients and six different subpopulations of RBCs were isolated. Fractions representing the most dense 0.8% of cells, the least dense 52.3% of cells, and cells with densities intermediate between these two extremes were analyzed. The deformability profile of the whole blood sample from which these different density fractions were isolated is represented by the dashed line. Density-fractionated RBCs from four other normal donors exhibited similar deformability profiles.

Osmotic deformability profiles of rabbit RBCs are shown in Fig 1. DI at a constant shear stress was determined as a function of suspending solution osmolality. These data indicate a loss of cellular deformability for rabbit RBCs as a function of cell age. Two distinct cellular factors account for the decreased DI of these cells. First, there is a decline in the maximum DI as the cells age, which is presumably due to a loss of surface area (see below); this change occurs throughout the cell’s lifespan. Second, the hypertonic arm of the deformability profile is shifted to the left (decreasing osmolality values), indicating dehydration as the cell ages. (This shift is due to the increased intracellular viscosity resulting from the dehydration.) Increased cellular dehydration with increasing cell age was independently confirmed by measuring cell hemoglobin concentration distribution profiles of cohorts of cells of different ages (Fig 2). There was a progressive increase in hemoglobin concentration with increasing cell age. Cell density analysis using discontinuous stractan density gradients confirmed the finding of a progressive increase in hemoglobin concentration with increasing cell age (data not shown).

Deformability profiles of aging mouse RBCs are shown in Fig 3. Here again, there was a progressive loss of surface area and increased cellular dehydration as the cells aged in vivo. It should be noted that the extent of dehydration (leftward shift of the hypertonic arm of the deformability profile) was not as large as that observed for the rabbit RBCs. Cell density analysis confirmed the finding of a lesser extent of dehydration of aging mouse RBCs (data not shown). This result probably reflects different distributions of cell age within the rabbit and mouse samples due to the different methods of preparation.

Osmotic deformability profiles for density-fractionated human RBCs are illustrated in Fig 4. With increasing cell density there was a progressive decrease in maximum DI and a leftward shift of the hypertonic arm of the deformability profile, characteristics similar to those seen for the aging rabbit or mouse RBCs. The maximum DI for the most dense cell fraction (0.8% of total cells) is approximately 68% of control, a value quite similar to that observed for the most aged rabbit RBCs. However, the absolute magnitude of the osmolality shift for the human cells is considerably greater than that observed for either the rabbit or the mouse RBCs. This result is expected because in the case of the human cells we have specifically selected for cells with the highest concentration of hemoglobin, i.e., the most dehydrated cells.

**Micropipette Measurements**

Aged cohorts of cells from rabbit. The distribution of surface areas within a given RBC population was well-fit by a log-normal distribution. Fitted distributions for the Day₀, whole blood sample and for aged cohorts for Day₁₀, Day₂₀, and Day₃₀ are shown in Fig 5. The decrease in cellular surface area with increasing age is clearly evident (Table 1). If we treat each cell as a separate observation, all of the mean values for area (except the Day₀ cohort and the controls) are significantly different from each other at the 0.99 confidence level as assessed by the Student’s t-test. However, if we recognize that there may be differences between different samples of the same type and treat each sample as a separate observation, only the areas and volumes of the Day₁ (n = 3) and Day₃₀ (n = 4) cohorts are significantly different. The areas of the Day₃₀ cohorts were also significantly smaller than the areas of the Day₀ whole blood samples. Note that the distribution of areas within a cohort was broad for all aged samples, that the decrease in

![Fig 4. Osmotic deformability profiles of normal, density-fractionated human RBCs.](image-url)
PROPERTIES OF SENESCENT RED CELLS

A progressive loss of cellular volume is evident. See Fig 5 legend for symbols.

All measurements are expressed as mean ± SD for all cells measured. Statistical comparisons for rabbit data are based on number and means of individual samples. Statistical comparisons for human data based on number of cells.

*Mean significantly different from Day, cohort (P < .01, Student's t-test).
†Human control sample was unfractionated blood.
‡Mean significantly different from control (P < .01, Student's t-test).
§Variance significantly different from control (P < .01, "F" test).
¶Variance significantly different from control (P < .05, Student's t-test).

Fig 6. Analysis of cellular volume by micropipette method. Fitted distribution curves for the volume data were generated similarly to those in Fig 5. Distributions are shown for the various aged samples from a total of 11 rabbits (three at Day, four at Day, and four at Day, and the Day whole blood sample (results pooled from four different rabbits). A progressive loss of cellular volume is evident. See Fig 5 legend for symbols.

### Table 1. Micropipette Measurements of Different Populations of Erythrocytes

<table>
<thead>
<tr>
<th>RBC Sample</th>
<th>No. of Cells (samples)</th>
<th>Cell Area (μm²)</th>
<th>Volume (μm³)</th>
<th>Sphericity</th>
<th>A/V (μm⁻¹)</th>
<th>Minimum Cylindrical Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control‡</td>
<td>421 (4)</td>
<td>96.7 ± 10.2‡</td>
<td>60.9 ± 12.0</td>
<td>0.77 ± 0.06</td>
<td>1.62 ± 0.21</td>
<td>2.74 ± 0.40</td>
</tr>
<tr>
<td>Aged; Day</td>
<td>270 (3)</td>
<td>97.8 ± 13.6‡</td>
<td>64.9 ± 14.3‡</td>
<td>0.80 ± 0.05</td>
<td>1.54 ± 0.16</td>
<td>2.91 ± 0.37</td>
</tr>
<tr>
<td>Aged; Day</td>
<td>372 (4)</td>
<td>93.5 ± 13.9</td>
<td>63.2 ± 15.3</td>
<td>0.81 ± 0.05</td>
<td>1.52 ± 0.18</td>
<td>2.97 ± 0.38</td>
</tr>
<tr>
<td>Aged; Day</td>
<td>427 (4)</td>
<td>86.5 ± 12.7</td>
<td>55.8 ± 12.6</td>
<td>0.81 ± 0.06</td>
<td>1.58 ± 0.19</td>
<td>2.88 ± 0.48</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control†</td>
<td>65 (1)</td>
<td>135 ± 10</td>
<td>93 ± 12</td>
<td>0.73 ± 0.02</td>
<td>1.59 ± 0.12</td>
<td>2.70 ± 0.21</td>
</tr>
<tr>
<td>Light fraction (MCHC ~ 31 g/dL)</td>
<td>77 (1)</td>
<td>135 ± 12</td>
<td>95 ± 14</td>
<td>0.74 ± 0.03</td>
<td>1.56 ± 0.13</td>
<td>2.77 ± 0.26</td>
</tr>
<tr>
<td>Densest fraction (MCHC &gt;37 g/dL)</td>
<td>76 (1)</td>
<td>112 ± 9$</td>
<td>70 ± 13§</td>
<td>0.73 ± 0.06</td>
<td>1.77 ± 0.23§</td>
<td>2.46 ± 0.38§</td>
</tr>
</tbody>
</table>

### Table 2. Membrane Elasticity of Different Aged Cohorts of Rabbit Erythrocytes

<table>
<thead>
<tr>
<th>Membrane Elasticity (μ)</th>
<th>Control</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day,</td>
<td>0.00554 ± 0.00117 (n = 11)</td>
<td>0.00593 ± 0.00087 (n = 11)</td>
</tr>
<tr>
<td>Day,</td>
<td>0.00541 ± 0.00123 (n = 11)</td>
<td>0.00492 ± 0.00053 (n = 9)</td>
</tr>
<tr>
<td>Day,</td>
<td>0.00548 ± 0.00067 (n = 10)</td>
<td>0.00555 ± 0.00070 (n = 11)</td>
</tr>
</tbody>
</table>

Data are listed as mean ± 1 SD. Controls are whole blood samples from each of the three rabbits tested.
measured with the same pipette. The Day$_1$ and Day$_5$ samples were measured on the same day with the same pipette and so can be compared directly. The Day$_{60}$ samples were measured on a different day with a different pipette, and so could not be compared directly with the other samples. So that direct comparisons could be made among the moduli of the different aged cohorts, we made the assumption that the actual moduli for the control samples were all the same. The value of the pipette radius for the pipette and the moduli of the different aged cohorts, we made the samples.

Density-fractionated human cells. The surface areas and volumes of human RBCs separated by density gradient centrifugation were also measured. In this case, the distributions of surface area and volume as well as sphericity were approximately Gaussian. When the densest 0.8% of the cells were compared with either whole blood or with lighter cell fractions, a statistically significant decrease in both the cell area and the cell volume were observed ($P < .001$) (Table 1). Consistent with the decrease in cell size, the ratio $A/V$ was larger for the densest cells, compared with either of the other two samples ($P < .001$). However, the mean value for the sphericity of the densest cells was not significantly different from the other two samples. In addition, the variance of the distribution of sphericities in the densest fraction was significantly greater than the variance of the whole blood or middle fractions (Fig 7). This broadening was consistent with, and far more evident than, the slight broadening in the distribution of sphericities of the oldest of the labeled cohorts of cells from the rabbit. Finally, the minimum cylindrical diameter through which each cell could pass was also calculated. The most dense cells, on average, could pass through cylinders significantly smaller than the less dense cells, an ability attributable to the smaller size of the most dense cells.

**DISCUSSION**

The data presented here document that rabbit and mouse erythrocytes become less deformable as they age. This decrease in deformability can be attributed to two factors, loss of surface area and dehydration. The surface area loss in the aged rabbit cells was independently documented with micropipette measurements that showed a surface area loss of approximately 10.5% for Day$_{60}$ RBCs from the rabbit when compared with a control population of cells or a loss of 11.6% compared with cohorts recovered on Day$_1$. Cellular dehydration is evident in the decrease in cell volume, the increase in MCHC, and the leftward shift of the deformability curves from the ektacytometer.

Also analyzed by ektacytometry and the micropipette method were density-fractionated human erythrocytes. The most dense cells showed a loss of deformability, surface area, and volume in a pattern similar to that seen for the aged cells isolated from both rabbits and mice. These data on the density fractionated cells agree closely with those of Nash and Wyard$^{28}$ and Linderkamp and Meiselman.$^{7}$ The first group reported losses of 8% in surface area and 11% in volume when comparing the densest and lightest 10% of the cells. The second group found losses of 12% in area and 20% in volume when comparing the densest 5% of cells with unFractionated blood. These decreases are slightly smaller than those found in the present study (17% in area, 25% in volume) in which the most dense 1% of cells was tested. These differences among the studies are most likely due to differences in the stringency of the isolation protocols. In the two previous studies it was concluded that the ratio of surface to volume did not change appreciably with cell age, and our finding that the sphericity of the cells did not change with age is in essential agreement with these results. The increase in $A/V$ observed in the present study reflects the decreased size of the most dense cells. (Recall that $A/V$ is a dimensional quantity that increases as particle size decreases.) This slight difference between present and previous results may be due to the smaller size of the cells obtained via the more stringent isolation protocols used in the present study, or to small systematic differences in measurement. Consistent with previous findings,$^{28}$ we found no change in the intrinsic elasticity of the membrane of biologically aged cells from the rabbit. Linderkamp and Meiselman$^{7}$ found an increase in the apparent membrane viscosity for the densest cells. The increase is due largely to the increased cell hemoglobin concentration.$^{27}$ Thus, it is likely that similar increases would have been observed had this parameter been measured in the present study.

The mechanism for the loss of surface area with RBC aging is unknown. The loss may be due to pinching off of membrane as the cells pass through the spleen, as is known to happen with membrane containing Heinz bodies. A second possibility is that membrane is lost as the result of breaking adhesive contacts between the RBC and reticuloendothelial cells. Such contacts might arise as the result of
recognition of bound antibody or complement components on the cell surface with the actual membrane loss occurring by a mechanical mechanism analogous to the formation of membrane "tethers" in fluid shear fields. The loss of cellular volume may be a consequence of the loss of membrane area. For example, if the membrane area became too small to enclose the cell volume within the constraints of a small aperture or stenosis in the microcirculation, large membrane tensions and trans-membrane pressures could be generated, sufficient to cause loss of cell contents.

The physiologic significance of the loss of deformability for the aged RBCs is difficult to quantitate. However, attempts at evaluating the impact of this change are best approached by examining other systems in which a loss of deformability is due to similar changes in cellular factors, namely, loss of surface and cell dehydration. RBCs in hereditary spherocytosis come closest to meeting this criterion. Recent studies have shown that the severity of the hemolytic process in hereditary spherocytosis is related to the extent of spectrin deficiency of the membrane, which in turn is directly related to the extent of cell surface area loss. Extrapolation of these deformability data to our observations suggests that the observed decrease in surface area may be of sufficient magnitude to contribute to sequestration and removal of aged cells from circulation. However, the similarity in minimum cylindrical diameter for all of the aged cohorts from rabbit and the fact that the mean minimum diameter for the most dense human cells is slightly but significantly smaller than control suggest that loss of deformability may not by itself account for the immediate removal of cells from the circulation. The immediate events leading to cell removal remain unknown and need to be further explored.

The present results have implications with regard to the current controversy over whether or not separation of cells based on their density is an effective method for obtaining aged cells. Clearly, the geometric changes that occur in cells as they age are also evident in the most dense human cells obtained by density fractionation. In addition, it has been found that membranes of these isolated human RBCs had significantly higher protein 4.1a to 4.1b ratios (Mohandas, unpublished observations), a biochemical feature of in vivo aged RBCs. This is in contrast to a variety of recent studies that have compared the biochemical properties of dense cells with old cells obtained with the aging models used in the present study. Few similarities in the biochemical parameters of dense cells and old cells have been found, leading many investigators to question the validity of density fractionation as a method for obtaining aged cells. In some instances, the failure to find characteristics of aged cells in dense populations may be due to less stringent density fractionation protocols. Several studies have compared the most dense 10% of cells with the least dense 10%, whereas we have restricted our measurement to the most dense 1%. However, the failure to find age-related changes in dense cells cannot always be attributed to the fractionation protocol. In a recent study in one of our own laboratories it was shown that even in the most dense 1% to 2% of cells from the rabbit, the enrichment of cells over 50 days of age was less than twofold to threefold over the circulating fraction. In reconciling these observations it may be important to distinguish between cells that are chronologically old and cells that (whatever their age) exhibit properties or behaviors characteristic of old cells. Our data show that as cells age, they become more dense. This is almost certainly a stochastic process, and not all cells may increase in density at the same rate. Thus, the densest cell fraction will include cells that are different ages chronologically, but exhibit rheologic properties characteristic of old cells.

Although the present studies do not directly address the immediate cause of senescent cell removal, we speculate that the increased MCHC that occurs with aging may lead indirectly to cell removal. Crosslinking of band-3 molecules by hemoglobin and the formation of "senescent" antigen could be facilitated by increased MCHC. In the most dense cells, formation of hemichromes and subsequent oxidative damage to membrane transport proteins could ultimately lead to increased membrane permeability and the loss of volume regulation. Such a mechanism is supported by the broad range of sphericities we have observed in the most dense human cells, which are known to be within days or hours of removal from the circulation.

**CONCLUSION**

The aging process for rabbit and mouse erythrocytes results in significant changes in the rheologic properties of the cell. The present results show that cell aging involves a progressive loss of surface area and volume and an increase in cell hemoglobin concentration. Although significant changes in these parameters can be tolerated without the immediate removal of the cell from the circulation, it is likely that these factors contribute to the ultimate demise of the cell. Identification of the immediate cause of cell removal must await further investigation.

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