CONCISE REPORT

Dense (Aged) Circulating Red Cells Contain Normal Concentrations of Adenosine Triphosphate (ATP)

By Francis H. Kirkpatrick, Ann G. Muhs, Ray K. Kostuk, and Conger W. Gabel

The densest 0.1%–1% of circulating red cells were separated from fresh blood, and the ATP content of a representative sample of such cells was determined. The dense ("old") cells had decreased amounts of ATP relative to unfractonated cells. However, the dense cells were also smaller, and the concentration of ATP in these cells was the same as in controls. Therefore, it seems unlikely that loss of cellular ATP is a causative factor in removal of senescent red cells from the circulation.

The red cell has a rather definite lifespan in the circulation (about 120 days ± 20 in man), but the process that determines this lifetime is unknown. Decrease in intracellular ATP has been proposed as a cause of destruction of senescent erythrocytes, and is attractive as a hypothesis because decline in cellular ATP is believed to be causative in removal of cells from banked blood and in pyruvate kinase deficiency. We have recently developed a technique for the determination of the ATP content of individual red cells. This allows simultaneous measurement of the content of ATP in a small population of cells and of the distribution of ATP content within that population. This technique has been applied to density-fractionated red cells to see if ATP is systematically altered in such cells.

MATERIALS AND METHODS

A detailed description of the technique for measuring the ATP content of single cells is published elsewhere. The essence of the technique, which is a refinement of the method originally described by Weed and Bessis and Lejeunie, is the lysis of a red cell by a laser pulse in the presence of firefly luciferin and luciferase. Photomissions from reaction of ATP with luciferase are counted, and photon counts are correlated with cellular ATP as measured by enzymatic analysis on bulk samples.

Dense cells were separated in two steps. Fresh blood from normal donors was centrifuged according to Smith on step gradients of Hypaque (sodium metrizoate), adjusted to only allow the densest 0.5%–1% of cells to sediment (14.03 vol of 5 mM Tris-150 mM NaCl, pH 7.4, plus 15.97 vol of 50% Hypaque [sodium diatrizoate]) or all but the lightest 5% to sediment (15.95 vol Tris NaCl plus 14.05 vol 50% Hypaque). The separated cells were washed in isotonic saline and then sealed into 2.7-mm (ID) polyethylene (Clay-Adams PE 320) tubing and centrifuged according to Murphy. The bottom or top 3%–5% of the cells were collected from such tubes by slicing the tubing below a clamped hemostat and...
were used directly for experiments. Control cells were processed in the same way but remixed after each step.

Mean cellular hemoglobin content was obtained by measuring hemoglobin with Drabkin's reagent\textsuperscript{10} and cell counts with a Celloscope. Mean cell volume was obtained by cell sizing on the Celloscope. It is known that electronic cell sizing tends to overestimate the volume of rigid cells.\textsuperscript{11,12} We expect the dense cells to be more rigid,\textsuperscript{13} but correction for this effect apparently is small (see below).

RESULTS

Histograms of photon counts and of apparent (electronic) cell volume are shown in Fig. 1. Within experimental uncertainty, the curves for dense, control, and light cells have the same overall shape and differ only in their mean value. In particular, there is no major "tail" on the low end of the photon count curve for dense cells. This observation virtually eliminates the possibility that the dense fraction consists of two populations, one with normal ATP content and one significantly depleted in ATP. If there is a separate "depleted" population, it must constitute less than about 5% of the "dense" cells.

The photon counts are converted into ATP contents using a calibration curve (Fig. 2). This curve was obtained by inhibiting red cell ATP synthesis with iodoacetamide, incubating at 37\degree C, and at intervals measuring total ATP by a bulk method (Sigma UV-361), and photon count distribution on a simultaneous sample. Details of the calibration procedure are given in reference 5. There is some variation in slope and intercept among donors;\textsuperscript{5} the curves in Fig. 1 and Fig. 2 are from the same donor.

Using the calibration curve, photon counts that have an approximately normal distribution can be converted into ATP contents; and ATP contents can be converted into ATP concentrations by the use of the cell sizing data. These calculations are shown in Table 1. The major result of such calculations is that the dense cells have ATP contents significantly below those of controls. However, when

![Fig. 1. Distribution of photon counts and cell-counter channels ("volume") in fractionated erythrocytes. The relative frequencies of photon counts and of channel number for erythrocytes, fractionated as described in the text, are shown. Vertical axes are adjusted for each sample to give comparable heights and are arbitrary. (\(\bullet\)) Dense cells; (\(\ast\)) processed cells; (\(\Delta\)) least dense cells.](image-url)
Fig. 2. Calibration of mean photon counts versus bulk cellular ATP. Unfractionated erythrocytes were treated with iodoacetamide and incubated at 37°C. Aliquots were removed at intervals and chilled to 4°C to stop ATP loss. Bulk samples were assayed by a 3-phosphoglycerate-coupled ATP assay (Sigma Chem. Corp. #366-UV), and single cell counts were obtained by laser lysis as previously described.

the decreased volume and increased hemoglobin concentration of the dense cells are corrected for, then the ATP concentrations of the dense cells are identical with those of controls. Since it seems unlikely that ATP concentration increases with cell age, we infer that the electronic measurement has not significantly overestimated cell volume in these experiments.

### Table 1. Hematologic Parameters and ATP Content of Fractionated Red Cells.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Densest Cells</th>
<th>Control Cells</th>
<th>Lightest Cells</th>
</tr>
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<tbody>
<tr>
<td>Number of cells*</td>
<td>60</td>
<td>32</td>
<td>62</td>
</tr>
<tr>
<td>Mean photon counts ± SE</td>
<td>48,000 ± 3,100</td>
<td>70,000 ± 4,500</td>
<td>81,000 ± 2,400</td>
</tr>
<tr>
<td>Mean cell volume (femtoliters)†</td>
<td>70.2</td>
<td>89.1</td>
<td>105.3</td>
</tr>
<tr>
<td>Mean hemoglobin/cell (g/liter cells)‡</td>
<td>432</td>
<td>344</td>
<td>335</td>
</tr>
<tr>
<td>ATP content (mole/cell, x 10⁻¹⁵)§</td>
<td>9.4</td>
<td>12.6</td>
<td>14.3</td>
</tr>
<tr>
<td>ATP concentration (mmole/liter cells)¶</td>
<td>1.34</td>
<td>1.41</td>
<td>1.36</td>
</tr>
<tr>
<td>ATP concentration (mmole/liter cell water)¶</td>
<td>1.96</td>
<td>1.98</td>
<td>1.80</td>
</tr>
</tbody>
</table>

*Measured by the single-cell technique.
†Measured on a Celsoscope.
‡Hemoglobin measured according to Dacie and Lewis.¹⁰
§Using calibration curve (Fig. 2).
¶Calculation of hemoglobin volume using a partial specific volume of 0.73, and subtraction of that volume from cell volume.
ATP CONCENTRATION OF DENSE RED CELLS

DISCUSSION

The results obtained in this study appear to be sufficiently strong to exclude models of red cell senescence in which a gradual decline of ATP is a causative factor. They also provide severe constraints on models in which ATP declines catastrophically towards the end of the cell’s lifespan. The initial fractionation on a density step gradient should be quite reliable, so that the “dense” cells after the second centrifugation are sampled from the densest 3% of the cells, as in the experiment shown. The efficiency of fractionation by the Murphy technique with previously fractionated cells is unknown. If it were perfectly efficient, the cells studied (the bottom 3% of a Murphy gradient in the experiment shown) would represent the densest 0.03% of the cells. Since about 1% of circulating red cells are eliminated each day, it would seem very unlikely that red cell ATP concentration decreases substantially before the cells are eliminated from the circulation. On the other hand, suppose that the second fractionation were completely inefficient. Then it would still be the case that cellular ATP concentration was normal until approximately one day before the removal of the cells, if, in fact, “dense” cells are senescent. It is difficult to see how decline in cellular ATP could cause removal of cells from the circulation, unless it were very abrupt. An abrupt decline in ATP would appear to require intervention of some other mechanism. Hence, if dense cells are actually the most senescent, then loss of cellular ATP appears to be unlikely as a cause of cellular senescence.

On the other hand, if dense cells swell before removal from the circulation, then the densest cells will not be the most senescent, even though they could be among the oldest cells. In that case, none of the presently used techniques of red cell fractionation can separate the most senescent cells, and hence, it becomes very difficult to test any proposed mechanism of red cell aging.

ACKNOWLEDGMENT

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REFERENCES

11. Grover NB, Naaman J, Ben-Sasson S, Doljanski F: Electrical sizing of particles in


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