Red Cell Age-Related Changes of Hemoglobins A₁a+b and A₁c in Normal and Diabetic Subjects

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ABSTRACT The minor hemoglobin components, hemoglobin A₁a+b and hemoglobin A₁c, were measured in the 10% youngest and 10% oldest erythrocytes of 15 normal and 14 diabetic subjects. Erythrocyte fractions were obtained by centrifugation in isopyknic concentrations of dextran: 28.5% of 40,000-mol wt dextran yielded the 10% lightest or young cells, and 30.5% dextran provided the 10% heaviest or old erythrocytes. Both normal and diabetic erythrocytes contain increased amounts of Hb A₁a+b and Hb A₁c in old as compared to young cells. In normal subjects, young cells contained 1.2±0.2%, and old cells contained 1.8±0.4% Hb A₁a+b. Corresponding values for diabetic cells were 1.7±0.6 and 2.6±0.9%. Hb A₁c increased from 3.1±0.8 to 6.0±1.1% in normals and from 5.1±2.1 to 10.1±3.7% in diabetics. The results indicate that both cell age and diabetes are significant determinants of the amounts of Hb A₁a+b and Hb A₁c.

INTRODUCTION

Microheterogeneity of hemoglobin in adult erythrocytes, in addition to the genetically determined A, A₂, and F, includes a number of minor components separable by chromatography on the cation exchange resin, Amberlite IRC 50 (Rohm and Haas Co., Philadelphia, Pa.). Allen et al. (1) and Clegg and Schroeder (2) designated these A₁a+b in the order of their elution ahead of the major hemoglobin A fraction, A₁c. Hemoglobins A₁α and A₁β are difficult to resolve; they account for 1–2% of total hemoglobin and have not been characterized chemically. Hb A₁c comprises 4–6% of the hemoglobin in normal adult erythrocytes. It differs from Hb A in having a hexose group attached as a ketoamine at one or both N-terminal valines of the normal β-chain (3–6). Rahbar et al. (7) first reported a twofold increase of Hb A₁c in diabetic patients.

We undertook this study to determine the effect of in vivo erythrocyte aging on the concentrations of Hb A₁a+b and Hb A₁c in normal volunteers and in subjects with diabetes mellitus.

METHODS

40-ml blood samples were obtained from 15 normal individuals with no family history of diabetes and from 14 diabetics. Erythrocyte fractions of the 10% least dense and 10% most dense were separated by isopyknic centrifugation in dextran as described by Phutrakul (8). Dextran T40 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. (Lot numbers 1600 and 2771). Stock solutions of 35% dextran were prepared weekly and densities were measured by pyknometry. Dilutions of the stock solution with normal saline were freshly prepared and used as separation media; 28.5% (wt/vol) dextran (density = 1.109 g/ml) and 30.5% dextran (density = 1.116 g/ml) were found to be most appropriate to obtain optimal erythrocyte separation. 3–4 ml of washed, packed erythrocytes were layered over 25 ml of dextran solution and centrifuged at 10,000 rpm for 1 h at 25°C in a Sorvall centrifuge equipped with an HB-4 swinging-bucket rotor (Ivan Sorvall, Inc., Norwalk, Conn.). To obtain sufficient numbers of erythrocytes at the chosen dextran densities, three tubes using the 28.5% dextran medium and one with the 30.5% medium were required.

During centrifugation in 28.5% dextran, erythrocytes corresponding to the 10–15% youngest (lightest) erythrocytes floated to the top of the centrifuged media in a discontinuous dark layer. In the 30.5% dextran, erythrocytes corresponding to the 10–15% oldest (heaviest) erythrocytes settled to the bottom in a dark layer. After centrifugation, the supernatant medium was removed and the erythrocytes were aspirated. Whole unfractonated erythrocytes were also studied. Reticulocyte counts were obtained on all young, whole, and old cell fractions.

After separation, the young and old cells were washed repeatedly in saline to remove dextran. Washed erythrocytes were hemolyzed in distilled water. Erythrocyte stroma were
removed by shaking vigorously in carbon tetrachloride and centrifuging at 3,000 rpm for 5 min. Hemoglobin separation was done by a modification of the method described by Trivelli et al. (9). The hemolysates were dialyzed overnight at 4°C against developer no. 6 (4.59 g of monosodium phosphate monohydrate, 1.18 g of anhydrous disodium phosphate, and 0.65 g of potassium cyanide per liter of water). Bio-Rex 70 columns (1 × 30 cm, Bio-Rad Laboratories, Richmond, Calif.) for chromatography were equilibrated with developer no. 6 initially for several days and subsequently with at least a 500-ml effluent volume between runs. All runs were performed at room temperature. Hemoglobin solutions of 25–50 mg were applied to the top of a column after the top 1 cm of resin layer was stirred, and elution with developer no. 6 was performed at 20–40 ml/h. After elution of the minor components, the major component (Hb A1C) was eluted with a stronger phosphate buffer (14.35 g of monosodium phosphate monohydrate and 6.52 g of anhydrous disodium phosphate per liter of water). 2-ml fractions were collected during initial development, and then 12-ml fractions were collected in a fraction collector. Fractions corresponding to A1b, A1c, and A1C were pooled separately, and hemoglobin concentrations were read at 410 nm in a Zeiss spectrophotometer (Carl Zeiss, Inc., New York). The proportions corresponding to each component were then calculated as a percentage of the total hemoglobin eluted. Hemoglobin A1C was eluted in the next 100–150 ml, after which Hb A1C was eluted with 100–200 ml of the stronger phosphate buffer. Good separation of Hb A1C, A1B, and Hb A1C was obtained.

### TABLE I

**Amounts of Minor Hemoglobin Components in Control and Diabetic Subjects**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Young RBC</th>
<th>Whole RBC</th>
<th>Old RBC</th>
<th>Young RBC</th>
<th>Whole RBC</th>
<th>Old RBC</th>
<th>Plasma sugar*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. K.</td>
<td>21</td>
<td>F</td>
<td>1.1</td>
<td>1.3</td>
<td>1.5</td>
<td>4.1</td>
<td>5.2</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>B. M.</td>
<td>25</td>
<td>M</td>
<td>1.0</td>
<td>1.3</td>
<td>1.6</td>
<td>2.0</td>
<td>3.9</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>A. S.</td>
<td>28</td>
<td>M</td>
<td>1.0</td>
<td>1.2</td>
<td>1.3</td>
<td>3.3</td>
<td>4.2</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>L. M.</td>
<td>29</td>
<td>F</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
<td>2.1</td>
<td>4.0</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>J. F.</td>
<td>29</td>
<td>M</td>
<td>1.0</td>
<td>1.2</td>
<td>1.3</td>
<td>2.6</td>
<td>3.8</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>J. L.</td>
<td>30</td>
<td>F</td>
<td>1.0</td>
<td>1.3</td>
<td>1.3</td>
<td>2.4</td>
<td>3.1</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>A. D.</td>
<td>37</td>
<td>F</td>
<td>1.3</td>
<td>1.2</td>
<td>1.6</td>
<td>3.3</td>
<td>4.2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>M. C.</td>
<td>43</td>
<td>F</td>
<td>1.1</td>
<td>1.2</td>
<td>1.6</td>
<td>1.9</td>
<td>4.2</td>
<td>5.0</td>
<td>102‡</td>
</tr>
<tr>
<td>R. J.</td>
<td>46</td>
<td>M</td>
<td>1.0</td>
<td>1.3</td>
<td>1.7</td>
<td>2.8</td>
<td>3.8</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>R. K.</td>
<td>51</td>
<td>M</td>
<td>1.0</td>
<td>2.1</td>
<td>1.7</td>
<td>3.0</td>
<td>4.5</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>D. V.</td>
<td>57</td>
<td>F</td>
<td>1.0</td>
<td>2.0</td>
<td>1.9</td>
<td>3.0</td>
<td>5.4</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>M. K.</td>
<td>57</td>
<td>F</td>
<td>1.2</td>
<td>2.0</td>
<td>2.5</td>
<td>4.8</td>
<td>6.0</td>
<td>8.2</td>
<td>65‡</td>
</tr>
<tr>
<td>J. M.</td>
<td>66</td>
<td>M</td>
<td>1.5</td>
<td>2.0</td>
<td>2.5</td>
<td>3.7</td>
<td>6.1</td>
<td>6.8</td>
<td>79‡</td>
</tr>
<tr>
<td>E. T.</td>
<td>75</td>
<td>F</td>
<td>1.2</td>
<td>1.9</td>
<td>2.5</td>
<td>3.1</td>
<td>4.9</td>
<td>6.5</td>
<td>84‡</td>
</tr>
<tr>
<td>R. B.</td>
<td>81</td>
<td>M</td>
<td>0.7</td>
<td>2.2</td>
<td>2.2</td>
<td>3.8</td>
<td>5.0</td>
<td>6.9</td>
<td>105‡</td>
</tr>
<tr>
<td>Mean±SD</td>
<td></td>
<td></td>
<td>1.2±0.2</td>
<td>1.8±0.4</td>
<td>1.8±0.4</td>
<td>3.1±0.8</td>
<td>4.6±0.9</td>
<td>6.0±1.1</td>
<td></td>
</tr>
</tbody>
</table>

**Diabetics**

| S. M.   | 22  | F   | 1.3       | 2.7       | 2.8     | 3.7       | 8.0       | 9.9     | 45–422        |
| B. M.   | 22  | F   | 1.7       | 2.9       | 2.4     | 5.8       | 6.8       | 8.6     | 217–530       |
| H. C.   | 27  | F   | 1.4       | 1.3       | 1.3     | 3.5       | 4.4       | 5.5     | 75–84         |
| D. H.   | 33  | M   | 1.8       | 1.8       | 1.7     | 4.3       | 6.7       | 7.8     | 62–190        |
| J. J.   | 41  | M   | 1.4       | 2.1       | 2.3     | 2.0       | 5.5       | 6.5     | 20–256        |
| H. L.   | 43  | M   | 0.6       | 1.8       | 2.4     | 2.5       | 4.6       | 5.4     | 90–110        |
| F. M.   | 44  | M   | 1.1       | 2.6       | 2.4     | 3.8       | 10.0      | 12.5    | 195–400       |
| G. R.   | 47  | M   | 1.7       | 3.6       | 4.3     | 7.1       | 15.0      | 18.7    | 66–1,180      |
| J. C.   | 49  | M   | 1.6       | 2.7       | 2.5     | 5.4       | 9.4       | 10.0    | 247–367       |
| F. W.   | 50  | M   | 2.8       | 2.7       | 3.0     | 7.0       | 7.8       | 9.2     | 43–494        |
| G. H.   | 53  | M   | 2.5       | 2.8       | 2.7     | 6.9       | 9.4       | 10.1    | 133–165       |
| B. B.   | 58  | F   | 1.5       | 2.2       | 2.7     | 3.5       | 7.6       | 9.2     | 165           |
| L. C.   | 60  | M   | 1.8       | 3.1       | 3.0     | 6.4       | 12.8      | 14.7    | 260–297       |
| H. M.   | 68  | F   | 2.8       | 3.8       | 3.8     | 9.1       | 11.8      | 13.7    | 431–548       |
| Mean±SD |     |     | 1.7±0.6   | 2.6±0.7   | 2.6±0.9 | 5.1±2.1   | 8.6±3.1   | 10.1±3.7 |               |

RBC, red blood cells (erythrocytes).

* Range of 2–3 h postprandial values (mg/100 ml) during 3 mo before study.

‡ 2–3 h postprandial values at time of study.
RESULTS

The reticulocyte counts of both diabetic and nondiabetic young cell fractions were generally 7–10%, as compared with 1.0% for whole blood and < 0.2% for old cells, confirming the efficacy of this method of erythrocyte separation. The amounts of Hb A\textsubscript{1a+b} and Hb A\textsubscript{1c} as a percentage of total hemoglobin are presented in Table I for young, old, and unfractionated whole erythrocytes. The range of values for 2-3 h postprandial plasma sugars obtained during the 3 mo before this study are given for the diabetic subjects. Postprandial plasma sugars were measured in the four oldest controls; all were 105 mg/100 ml or less. F ratios calculated from analysis of variance of the data for young and old cells are given in Table II. Diabetes and erythrocyte age are highly significant (P << 0.0005) determinants of the amounts of both Hb A\textsubscript{1a+b} and Hb A\textsubscript{1c}. The interaction between cell age and diabetes approaches borderline significance for Hb A\textsubscript{1c}. None of the other interaction terms included in the residual, i.e., sex vs. cell age or sex vs. diabetes, is significant. No significant correlation was found for either minor hemoglobin fraction or for the age of the subject.

The highest values for Hb A\textsubscript{1c} were found in those diabetics who had the poorest control. Patient G. R. had the highest Hb A\textsubscript{1c} and also had the highest plasma glucose, 1.180 mg/100 ml, recorded 1 mo before this study. No other person had a recorded plasma glucose above 600 mg/100 ml. The four patients with the closest control over plasma glucose, H. C., D. H., J. J., and H. L., had the lowest values for Hb A\textsubscript{1c}. Interestingly, patient F. M. was studied during hospitalization for initial control of diabetes. His plasma glucose values had been 301–385 mg/100 ml. Insulin therapy was started and his 3-h postprandial glucose values during the preceding week had decreased considerably, ranging from 155 to 195 mg/100 ml. His young cells contained disproportionately small amounts of Hb A\textsubscript{1c} compared with his unfractionated and old cells, suggesting that recent exposure to smaller elevations in plasma glucose was associated with relatively less formation of Hb A\textsubscript{1c} in newly produced erythrocytes. Patient B. M., in contrast, after a period of fairly good control, had a viral infection during which her diabetes was clearly uncontrolled as measured by a fivefold increase in 24-h urine glucose 1–2 wk before the sample was obtained for this study. Her young cell Hb A\textsubscript{1c} was disproportionately increased.

DISCUSSION

Fractionation of erythrocyte samples based on increasing density with age (8, 10–14) has provided evidence for age-dependent decreases in water, lipid, activity of enzymes, and content of glycolytic intermediates. Erythrocyte age-related increase in hemoglobin oxygen affinity has also been reported (15). The results described here include an increase in the minor hemoglobin components A\textsubscript{1a+b} and A\textsubscript{1c} with erythrocyte age. In diabetes the increase is significantly greater than in normals and appears to be related to the degree of hyperglycemia. These findings suggest that post-translational changes in hemoglobin structure are part of in vivo senescence of the erythrocyte. Haney et al. (16) have reported the incorporation of \textsuperscript{57}Fe and \textsuperscript{14}C]leucine. Their results indicate that during the life-span of the erythrocyte, Hb A is slowly and irreversibly glycosylated.

Structural studies by Holmquist and Schroeder (3, 4), Bookchin and Gallop (5), and Bunn et al. (6) indicate that a hexose, most probably glucose, is attached to the N-terminal valine of the \&-chain of Hb A to form the stable ketoamine found in Hb A\textsubscript{1c}. Bunn and Briehl (17) have further shown that Hb A\textsubscript{1c} has a higher oxygen affinity than Hb A and is fourfold less responsive to the effect of 2,3-diphosphoglycerate in lowering oxygen affinity.

Koenig and Cerami (18) have reported a linear increase in the amount of Hb A\textsubscript{1c} in adult diabetic mice (C57BL/KsJ - db/db) beginning about 4 wk after the onset of hyperglycemia. They leave open the question of whether the chemical modification of Hb A proceeds via Hb A\textsubscript{1a+b} as intermediates or directly to Hb A\textsubscript{1c}. The increase in Hb A\textsubscript{1a+b} and Hb A\textsubscript{1c} in our diabetic subjects suggests that both participate and that the amounts reflect the adequacy of therapy as measured by

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Hb A\textsubscript{1a+b} F ratio</th>
<th>Probability</th>
<th>Hb A\textsubscript{1c} F ratio</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males vs. females</td>
<td>0.03</td>
<td>0.90 &lt; P &lt; 0.95</td>
<td>0.67</td>
<td>0.75 &lt; P &lt; 0.90</td>
</tr>
<tr>
<td>Diabetic vs. nondiabetic</td>
<td>18.54</td>
<td>P &lt;&lt; 0.0005</td>
<td>26.88</td>
<td>P &lt;&lt; 0.0005</td>
</tr>
<tr>
<td>Young vs. old cell age</td>
<td>20.11</td>
<td>P &lt;&lt; 0.0005</td>
<td>45.91</td>
<td>P &lt;&lt; 0.0005</td>
</tr>
<tr>
<td>Diabetes X cell age</td>
<td>0.89</td>
<td>0.25 &lt; P &lt; 0.50</td>
<td>3.20</td>
<td>0.05 &lt; P &lt; 0.10</td>
</tr>
<tr>
<td>Residual</td>
<td>0.23</td>
<td>0.50 &lt; P &lt; 0.75</td>
<td>0.51</td>
<td>0.75 &lt; P &lt; 0.90</td>
</tr>
</tbody>
</table>
postprandial blood sugar levels. Since the ketoamine binding to the β-chain is quite stable, it seems likely that changes in amount of Hb Aτε over relatively short times represent changes in cohorts of erythrocytes that have or have not been exposed to elevated levels of blood glucose.

Glycosylation of glomerular basement membranes in diabetics has been reported by Spiro (19) and Beisswenger and Spiro (20). Like et al. (21) have described light and electron microscopic glomerular changes in C57BL/Ks mice which mimic those found in human diabetes. The appearance and progression of these abnormalities coincide with the changes in Hb Aτε observed in the same strain of mice by Koenig and Cerami (18). In a series of papers (22–27) attempting to account for some of the pathologic abnormalities of diabetes, Ditzel and coworkers have presented evidence to suggest that oxygen transport may be impaired in diabetics and that hypoxic injury may account for the microangiopathy. A relationship between the Hb Aτε formation and tissue hypoxia is still speculative. Nonetheless, further study of in vivo chemical modification of Hb A and of the effects of minor hemoglobin components that result are of interest in understanding both normal erythrocyte senescence and diabetes.

Addendum. Since preparation of this paper, Haney and Bunn (28) have described both in vivo and in vitro glycosylation of Hb A to form Hb Aτε. Their results indicate that the reaction in vivo is slow, nonenzymatic, specific, and nearly irreversible.

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REFERENCES

23. Ditzel, J., H. Andersen, and N. Daugaard Peters. 1975. Oxygen affinity of haemoglobin and red cell 2,3-diphos-