Red Cell Age-Related Changes of Hemoglobins A_{Ia+b} and A_{Ic} in Normal and Diabetic Subjects

JAMES F. FITZGIBBONS, ROBERT D. KOLER, and RICHARD T. JONES

From the Divisions of Medical Genetics and Hematology and the Department of Biochemistry, University of Oregon Health Sciences Center, Portland, Oregon, 97201

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 Alath and Hb Ale in old as compared to young cells. In normal subjects, young cells contained $1.2\pm0.2\%$, and old cells contained $1.8\pm0.4\%$ Hb A1a+b. Corresponding values for diabetic cells were 1.7±0.6 and 2.6±0.9%. Hb Are increased from 3.1±0.8 to $6.0 \pm 1.1\%$ in normals and from 5.1 ± 2.1 to $10.1 \pm 3.7\%$ in diabetics. The results indicate that both cell age and diabetes are significant determinants of the amounts of Hb A1a+b and Hb A1e.

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 A_{1a-e} in the order of their elution ahead of the major hemoglobin A fraction, A₁₁. Hemoglobins A_{1a} and A_{1b} are difficult to resolve; they account for 1–2% of total hemoglobin and have not been characterized chemically. Hb A_{1e} 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 A_{1e} in diabetic patients.

We undertook this study to determine the effect of in vivo erythrocyte aging on the concentrations of Hb A_{Ia+b} and Hb A_{Ie} 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 $ar{\mathrm{T}}$ 40 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 unfractionated 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 hemolysed in distilled water. Erythrocyte stroma were

A preliminary abstract of this work appeared in Clin. Res. 24: 307A (Abstr.), 1976.

Received for publication 5 April 1976 and in revised form 8 June 1976.

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 $(1 \times 30 \text{ cm}, \text{Bio-Rat})$ and 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 AII) 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 A1a+b, A1e, and AII 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 AIa and AIb were eluted together in the first 35 ml of effluent volume. Hb A_{Ie} was eluted in the next 100-150 ml, after which Hb AII was eluted with 100-200 ml of the stronger phosphate buffer. Good separation of Hb A1a+b, Hb A1e, and Hb A₁₁ was obtained.

 TABLE I

 Amounts of Minor Hemoglobin Components in Control and Diabetic Subjects

				Hb AIa+b			Hb Aie		
Subject	Age	Sex	Young RBC	Whole RBC	Old RBC	Young RBC	Whole RBC	Old RBC	Plasma sugar*
Controls									
М. К.	21	F	1.3	1.2	1.5	4.1	5.2	6.3	
B. M.	25	Μ	1.3	1.6	1.6	2.0	3.9	5.0	
A. S.	28	Μ	1.0	1.4	1.3	3.3	4.2	5.6	
L. M.	29	F	1.2	2.2	1.9	2.1	4.0	5.4	
J. F.	29	Μ	1.0	1.3	1.3	2.6	3.8	5.5	
J. L.	30	F	1.6	1.7	1.2	2.4	3.1	4.0	
A . D.	37	F	1.3	1.6	1.6	3.3	4.8	6.5	
M. C.	43	F	1.2	1.8	1.6	1.9	4.2	5.0	102‡
R. J.	46	Μ	1.2	1.3	1.7	2.8	3.8	4.8	
R. K.	51	Μ	1.1	2.6	2.5	3.0	4.5	7.0	
D. V.	57	F	1.1	1.7	1.9	3.0	5.4	6.9	
M. K.	57	F	1.2	2.0	2.5	4.8	6.0	8.2	65‡
J. M.	66	Μ	1.5	2.0	1.9	3.7	6.1	6.8	79‡
E . T.	75	F	1.2	1.9	1.9	3.1	4.9	6.5	84‡
R. B.	81	Μ	0.7	2.2	1.8	3.8	5.0	6.9	105‡
$Mean \pm SD$			1.2 ± 0.2	1.8 ± 0.4	1.8 ± 0.4	3.1 ± 0.8	4.6 ± 0.9	6.0 ± 1.1	•
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
Н. С.	27	F	1.4	1.3	1.3	3.5	4.4	5.5	75-84
D. H.	33	F	1.8	1.8	1.7	4.3	6.7	7.8	62-190
J. J.	41	Μ	1.4	2.1	2.3	2.0	5.5	6.5	20-256
H. L.	43	Μ	0.6	1.8	1.0	2.3	4.6	5.4	90-110
F. M.	44	М	1.1	2.6	2.4	3.8	10.0	12.5	195-400
G. R.	47	Μ	1.7	3.6	4.3	7.1	15.0	18.7	66-1,180
J. C.	49	F	1.6	2.7	2.5	5.4	9.4	10.0	247-367
F . W.	50	Μ	2.8	2.7	3.0	7.0	7.8	9.2	43-494
G. H.	53	Μ	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	Μ	1.8	3.1	3.0	6.4	12.8	14.7	260-297
Н. М.	68	F	2.8	3.8	3.8	9.1	11.8	13.7	431-548
$Mean \pm SD$			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.

		Hb AIa+b	Hb AIc		
Source of variation	F ratio	Probability	F ratio	Probability	
Males vs. females	0.03	0.90 < P < 0.95	0.67	0.75 < P < 0.90	
Diabetic vs. nondiabetic	18.54	$P \ll 0.0005$	26.88	$P \ll 0.0005$	
Young vs. old cell age	20.11	$P \ll 0.0005$	45.91	$P \ll 0.0005$	
Diabetes \times cell age	0.89	0.25 < P < 0.50	3.20	0.05 < P < 0.10	
Residual	0.23	0.50 < P < 0.75	0.51	0.75 < P < 0.90	

TABLE II Analysis of Variance

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 AIa+b and Hb AIe 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 ($P \ll 0.0005$) determinants of the amounts of both Hb A1a+b and Hb A1e. The interaction between cell age and diabetes approaches borderline significance for Hb Are. 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 Are were found in those diabetics who had the poorest control. Patient G. R. had the highest Hb Are 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 Are. 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 Are 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 Are 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_{1e} 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 some 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_{1a+b} and A_{1e} with erythrocyte age. In diabetics 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 ⁵⁰Fe and [¹⁴C]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 β -chain of Hb A to form the stable ketoamine found in Hb A_{1e}. Bunn and Briehl (17) have further shown that Hb A_{1e} 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_{1e} 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_{1a+b} as intermediates or directly to Hb A_{1e}. The increase in Hb A_{1a+b} and Hb A_{1e} in our diabetic subjects suggests that both participate and that the amounts reflect the adequacy of therapy as measured by

postprandial blood sugar levels. Since the ketoamine binding to the β -chain is quite stable, it seems likely that changes in amount of Hb A₁₀ 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 Are 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 Are 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 A_{Ie} . Their results indicate that the reaction in vivo is slow, nonenzymatic, specific, and nearly irreversible.

ACKNOWLEDGMENTS

We thank Doctors John Stephens and Matthew Riddle for allowing us to study their patients. Phornsri Phutrakul instructed us in the method for separating erythrocytes, Marilyn Jones provided expert technical assistance, Alice J. Fitzgibbons gave invaluable laboratory assistance and manuscript advice, and Marian Rivas verified the statistical analyses.

This work was supported in part by research grant AM 18006 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

REFERENCES

- 1. Allen, D. W., W. A. Schroeder, and J. Balog. 1958. Observations on the chromatographic heterogeneity of normal adult and fetal human hemoglobin: A study of the effects of crystallization and chromatography on the heterogeneity and isoleucine content. J. Am. Chem. Soc. 80: 1628-1634.
- Clegg, M. D., and W. A. Schroeder. 1959. A chromatographic study of the minor components of normal adult human hemoglobin including a comparison of hemoglobin from normal and phenylketonuric individuals. J. Am. Chem. Soc. 81: 6065-6069.
- 3. Holmquist, W. R., and W. A. Schroeder. 1966. A new N-terminal blocking group involving a Schiff base in hemoglobin A_{1c}. *Biochemistry*. 5: 2489–2503.
- 4. Holmquist, W. R., and W. A. Schroeder. 1966. The

in vitro biosynthesis of hemoglobin A_{1e}. Biochemistry. 5: 2504–2512.

- Bookchin, R. M., and P. M. Gallop. 1968. Structure of hemoglobin A_{1e}: Nature of the N-terminal β-chain blocking group. *Biochem. Biophys. Res. Commun.* 32: 86-93.
- Bunn, H. F., D. N. Haney, K. H. Gabbay, and P. M. Gallop. 1975. Further identification of the nature and linkage of the carbohydrate in hemoglobin A_{1e}. Biochem. Biophys. Res. Commun. 67: 103-109.
- Rahbar, S., O. Blumenfeld, and H. M. Ranney. 1969. Studies of an unusual hemoglobin in patients with diabetes mellitus. *Biochem. Biophys. Res. Commun.* 36: 838-843.
- Phutrakul, P. 1976. Age-related changes in red cell activities of glycolytic enzymes, reduced glutathione, and hemoglobin denaturation. M.S. Thesis. University of Oregon Health Sciences Center, Portland, Oreg. 1-85.
- 9. Trivelli, L. A., H. M. Ranney, and H-T. Lai. 1971. Hemoglobin components in patients with diabetes mellitus. N. Engl. J. Med. 284: 353-357.
- Van Gastel, C. 1968-RVI. Methods for studying the in vivo aging of red cells. Blood Information Service, Buffalo, N. Y. 1-32.
- 11. Schulman, H. M. 1967. The fractionation of rabbit reticulocytes in dextran density gradients. *Biochim. Biophys. Acta.* 148: 251-255.
- Leif, R. C., and J. Vinograd. 1964. The distribution of buoyant density of human erythrocytes in bovine albumin solutions. *Biochemistry.* 51: 520-528.
- Keitel, H. G., H. Berman, H. Jones, and E. MacLachlan. 1955. The chemical composition of normal human red blood cells, including variability among centrifuged cells. *Blood.* 10: 370–376.
- Bernstein, R. E. 1959. Alterations in metabolic energetics and cation transport during aging of red cells. J. Clin. Invest. 38: 1572-1586.
- Edwards, M. J., and D. A. Rigas. 1967. Electrolyte-labile increase of oxygen affinity during in vivo aging of hemoglobin. J. Clin. Invest. 46: 1579-1588.
- Haney, D. N., H. F. Bunn, S. Kamen, K. Gabbay, and P. M. Gallop. 1975. Structural and biosynthetic studies of hemoglobin A_{1e}. *Blood.* 46: 1051. (Abstr.)
- Bunn, H. F., and R. W. Briehl. 1970. The interaction of 2,3-diphosphoglycerate with various human hemoglobins. J. Clin. Invest. 49: 1088-1095.
- Koenig, R. J., and A. Cerami. 1975. Synthesis of hemoglobin A_{1e} in normal and diabetic mice: Potential model of basement membrane thickening. *Proc. Natl. Acad. Sci. U. S. A.* 72: 3687-3691.
- Spiro, R. G. 1971. Glycoproteins and diabetic microangiopathy. In Joslin's Diabetes Mellitus. A. Marble, P. White, R. F. Bradley, and L. P. Krall, editors. Lea & Febiger, Philadelphia. 11th edition. 146-156.
- Beisswenger, P. J., and R. G. Spiro. 1970. Human glomerular basement membrane: Chemical alteration in diabetes mellitus. Science (Wash. D. C.). 168: 596-598.
- Like, A. A., R. L. Lavine, P. L. Poffenbarger, and W. L. Chick. 1972. Studies in the diabetic mutant mouse. VI. Evolution of glomerular lesions and associated proteinuria. Am. J. Pathol. 66: 193-224.
- 22. Ditzel, J. 1968. Functional microangiopathy in diabetes mellitus. *Diabetes*. 17: 388-397.
- 23. Ditzel, J., H. Andersen, and N. Daugaard Peters. 1975. Oxygen affinity of haemoglobin and red cell 2,3-diphos-

phoglycerate in childhood diabetes. Acta Paediatr. Scand. 64: 355-361.

- 24. Ditzel, J., and E. Standl. 1975. The oxygen transport system of red blood cells during diabetic ketoacidosis and recovery. *Diabetologia*. 11: 255-260.
- and recovery. Diabetologia. 11: 255-260.
 25. Ditzel, J., and E. Standl. 1975. The problem of tissue oxygenation in diabetes mellitus. II. Evidence of disordered oxygen release from the erythrocytes of diabetics in various conditions of metabolic control. Acta Med. Scand. Suppl. 578: 59-68.
- 26. Ditzel, J., and E. Standl. 1975. The problem of tissue

oxygenation in diabetes mellitus. I. Its relation to the early functional changes in the microcirculation of diabetic subjects. Acta Med. Scand. Suppl. 578: 49-58.

- 27. Ditzel, J. 1975. The problem of tissue oxygenation in diabetes mellitus. III. The "three-in-one concept" for the development of diabetic microangiopathy and a rational approach to its prophylaxis. Acta Med. Scand. Suppl. 578: 69-83.
- 28. Haney, D. N., and H. F. Bunn. 1976. The glycosylation of hemoglobin in vivo and in vitro. *Fed. Proc.* 35: 1606. (Abstr.)