Hemoglobin A₁ Quantity in Individual Red Cells of Normal Adults

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TAKI, K. *Hemoglobin A₁ Quantity in Individual Red Cells of Normal Adults.* *Tohoku J. exp. Med.*, 1976, 119 (1), 71-77 — HbA₁ weight in individual red cells, amounting to 13,746, was quantitatively determined in 72 male and 17 female healthy adults. The red cells were besmeared on the anti-HbA₁ serum agarose plate and the precipitation rings formed were measured from the size printed on sheets of printing paper. HbA₁ weight in individual cells was calculated from the calibration curve prepared specially for HbA₁ weight in a red cell and the volume of a precipitation ring. HbA₁ weight in individual cells ranged from 4.7 to 62.6 pg and averaged 23.7 pg. The mean HbA₁ weight in individual cells per head ranged from 18.1 to 34.0 pg and averaged 26.4 ± 4.3 pg. No difference in the mean HbA₁ weight was found between male and female adults, and among the age groups of them. Average ratio of the mean HbA₁ weight in individual cells to the total hemoglobin in the average red cell was 87.4%. The HbA₁ weight in the average red cell was determined from HbA₁ concentration and red cell counts of the whole blood. The correlation coefficient between the mean HbA₁ weight in individual cells and the HbA₁ weight in the average red cell was 0.546 ($p < 0.001$). ——— adult hemoglobin; red blood cells; immunoassay

The mean value of hemoglobin quantity in individual red blood cells is easily obtained from red cell count and hemoglobin concentration in the whole blood. But, it is difficult to measure directly hemoglobin in individual red cells, especially in each of the hemoglobin types. Katsura (1964) devised the microprecipitation method for detection of fetal hemoglobin (HbF) in individual red cells, and counted red cells containing fetal hemoglobin (HbF cells) in adult and umbilical cord blood.

Kamada (1969) measured diameters of precipitation rings of HbF cells in the blood of normal infants by the microprecipitation, and concluded that a cell forming a small precipitation ring contained a small amount of HbF. Gitlin et al. (1968) measured carbonic anhydrase B, β -chain and γ -chain hemoglobins in individual red cells by application of the microprecipitation.

Daufi and Rondell (1969) modified the microprecipitation method and developed cytoimmunodiffusion. Boyer et al. (1975a) examined the percentage of HbF cells in normal adults and others by the cytoimmunodiffusion, and found that HbF cells increased during a period of pregnancy and in the blood of leukemia.

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Headings et al. (1975) identified hemoglobins A₁, S and F within individual red cells by the cytoimmunodiffusion combined with fluorescent antibody technique.

Katsura et al. (1976) reported that a volume of precipitate in immunodiffusion related to an antigen weight. In this paper, the main component of adult hemolysate, HbA₂, in individual red cells of normal adult is quantitatively determined from the volume of precipitation ring formed in antiserum agarose plate.

MATERIALS AND METHODS

Preparation of anti-HbA₁ serum

HbA₁ was prepared by DEAE-sephadex column chromatography introduced by Huisman and Dozy (1965). HbA₁ fraction was concentrated and confirmed by immunoelectrophoresis. A goat was subcutaneously injected with a mixture of 20 mg HbA₁ and Freund's complete adjuvant, and with 30 mg HbA₁ once a week for 8 weeks. The crude anti-HbA₁ serum was absorbed with HbF and specified. Precipitin titer and content of the specific anti-HbA₁ antibody were determined by Ascoli's ring test with HbA₁ solution containing 36 g HbA₁ per 100 ml. The precipitin titer and content were 1 : 12,800 and 1 : 4, respectively.

Preparation of the calibration curve

Five metal discs 2 mm in diameter and 0.5 mm in thickness were prepared and pasted on a glass plate (12.5 × 2.5 cm) at equal distances on a straight line. The size of the metal disc corresponded to the size of about 250 times as large as a red cell. A glass tray (12.5 × 2.5 cm, 1.5 cm depth) was covered with the glass plate with the discs, and was filled with the antiserum-agarose mixture which was prepared by mixing 2 volumes of the anti-HbA₁ serum and 1 volume of 3% agarose normal saline solution at 55°C. HbA₁ concentration of stroma-free adult hemolysate was quantitatively determined by single radial immunodiffusion of Mancini et al. (1965), and was diluted to four grades with normal saline. Then, 1.4 μ l each of the diluted hemolysates and 1.6 μ l of the undiluted ones were applied in the wells molded on the anti-HbA₁ serum agarose plate. The applied HbA₁ weight was as follows: 33.8, 67.5, 145.3, 193.7 and 221.4 μ g. The anti-HbA₁ serum agarose plate was kept in a moist chamber at 4°C, and the diameters of precipitation rings were estimated every day until the growth of precipitation rings completely stopped.

After the antigen diffusion completely finished, the precipitation ring was divided vertically through the center of the well, and the vertical section of the precipitate was photographed. The shape of precipitate in the antiserum was hemispherical (Fig. 1), and the volume was calculated from $\frac{4}{3} \pi r^3 \cdot 1/2$.

Fig. 1. Top: Precipitation rings in a view from the upper surface of the anti-HbA₁ serum agarose plate with the wells corresponding to 250 times as large as a red cell. HbA₁ weight in the wells was 33.8, 67.5, 145.3, 193.7 and 221.4 μ g, from left to right. Bottom: The vertical section of the precipitation rings shown in the top.

Preparation of anti-HbA₁ serum agarose plate for microprecipitation

Two steepers 0.5 mm in thickness were pasted on a slide glass, 1.5 cm apart and parallel to each other, and a cover glass was put on them. Two volumes of anti-HbA₁ serum were mixed with one volume of 3% agarose normal saline solution at 55°C, and the mixture was poured in the space between the cover glass and the slide glass.

Quantitation of HbA₁ weight in individual red cells

One drop of about 0.01% red cell suspension was besmeared on the anti-HbA₁ serum agarose plate. Then the plate was inclined, and the excess suspension on the plate was absorbed with a piece of filter paper from its lower corner, and the plate was kept in a moist chamber at 4°C. The microprecipitation rings which appeared around the red cells were microscopically photographed with a phase contrast plate under the constant light. On the other hand, an objective micrometer was photographed microscopically with the same eyepiece and object lens. The photographs were printed on sheets of printing paper with a constant magnification, and the size of precipitation rings was estimated with the aid of the photograph of the microscale. The volume (x) of the precipitation ring was calculated from the formula $x = 4/3\pi r^3 \cdot 1/2$. HbA₁ in individual red cells was read on the calibration curve.

Quantitation of HbA₁ in the average red cell

HbA₁ weight in whole blood hemolysate was determined by single radial immunodiffusion, and HbA₁ weight in the average red cell was calculated from HbA₁ weight and red cell count.

RESULTS

HbA₁ weight in individual red cells of 89 healthy adults, 72 males and 17 females, was quantitatively determined and the red cells examined amounted to 13,746. The minimal and maximal counts of red cells per head were 66 and 303, respectively, and the average was 154.

HbA₁ weight determined by the microprecipitation ranged from 4.7 to 62.6 pg, and averaged 23.7 pg. The distribution of HbA₁ weight in individual cells showed quite a normal curve as shown in Fig. 2. The mean HbA₁ weight in individual cells per head ranged from 18.1 to 34.0 pg, and averaged 26.4 ± 4.3 pg (s.d.). The mean HbA₁ weight in individual cells of 72 males and 17 females were 26.1 ± 4.4 pg and 27.5 ± 3.8 pg, respectively. No significant difference was found between them. The 89 adults were divided into age groups at intervals of decade, but no significant difference in the mean HbA₁ weight was found among them, although it was slightly higher in the second decade than in the other decades.

HbA₁ weight in the average red cell calculated from HbA₁ concentration and red cell count of the whole blood ranged from 17.3 to 33.3 pg, and averaged 27.1 ± 3.3 pg. The relationship between the mean HbA₁ weight in individual cells and the HbA₁ weight in the average red cell was shown in Fig. 3. The correlation coefficient was 0.546 ($p < 0.001$).

The total hemoglobin in the average red cell ranged from 26.1 to 33.3 pg, and averaged 30.2 ± 1.5 pg. The rate of the mean HbA₁ weight in individual red cells and the HbA₁ weight in the average red cell to the total hemoglobin in the average

Fig. 2. The distribution pattern of the HbA₁ weight in individual red cells of 89 normal adults.

Fig. 3. The correlation diagram between the mean HbA₁ weight in individual red cells and the HbA₁ weight in the average red cell.

red cell were 87.4 and 88.5%, respectively. These results almost corresponded with the low boundary of HbA₁ percentage reported by Huisman and Dozy (1965).

DISCUSSION

The method of quantitative determination of HbA₁ in individual red cells

The microprecipitation rings printed were measured duplicately or triplicately, and the diameter was determined from the average. The negative images of precipitation rings were printed on sheets of printing paper with variation in exposure. The change in the ring size due to variation in exposure was not

observed except photographs printed extremely dark or pale.

Changes in size of the microprecipitation ring in the course of time were tested. The mean HbA₁ weight of 12 red cells observed during a period from 2 to 8 days after the application were as follows: 26.4 pg on the second day, and 26.5 pg on the fourth and eighth days. No significant difference was found among them.

After the microprecipitation ring was formed, the antiserum agarose plate was cut vertically, and a shape of the ring on the vertical section was observed (Fig. 4). The mean ratio of the depth to the diameter or the parallel line to the diameter was almost constant as follows: 2.00 ± 0.08 ($n=8$) on the second day, 1.99 ± 0.07 ($n=11$) on the fourth day, and 2.00 ± 0.77 ($n=10$) on the eighth day. Therefore, the volume of microprecipitation ring can be calculated from the diameter of the ring.

Fig. 4. Top: The microprecipitation rings in a view from the upper surface of the anti-HbA₁ serum agarose plate. The white spot on the round of the right microprecipitation ring was a dust particle.

Bottom: The vertical section of the microprecipitation ring.

HbA₁ weight and volume of the precipitate in the anti-HbA₁ serum agarose plate for preparation of the calibration curve were reduced to $(1/250)^3$ and graduated on a sheet of graph paper, because the wells on the antiserum agarose plate were 250^3 times as large in volume as the average red cell. And, the calibration curve was given as the formula $y=2217.0x-1943.7$, where y stands for volume of microprecipitation ring and x stands for HbA₁ weight in individual red cells. The reduced volumes ranged from 4×10^3 to $35 \times 10^3 \mu\text{m}^3$. The volumes of many precipitation rings were beyond the range. Therefore, HbA₁ of these rings was calculated on the line extended from the calibration line. This calculation is thought to be adequate, because Katsura et al. (1976) have reported that the volume of a precipitate in antiserum gel plate is related with the antigen weight in a wide range.

In conclusion, HbA₁ weight in individual cells can be determined with sufficient accuracy.

HbA₁ weight in individual red cells

HbA₁ in the present study is the main component in adult blood hemolysate, but not HbA₁ given by Huisman et al. (1958), who divided adult hemolysate into A₀, A₁ and A₂ by column chromatography. Gitlin et al. (1968) measured β -chain hemoglobin in individual erythrocytes by application of the microprecipitation, and obtained the result that hemoglobin ranged from 18.8 to 62.2 μg per cell. The maximal HbA₁ weight in this study and the maximal β -chain hemoglobin were nearly equal, but the minimal weight of HbA₁ was a quarter of the minimal β -chain hemoglobin per cell. This difference might be due to the fact that the precipitin titer of the anti-HbA₁ serum was higher than that of the anti- β -chain serum, because it is considered that β -chain hemoglobin is probably identical with HbA₁.

Red cells with a very small amount of HbA₁ probably contain other hemoglobin, because HbF cells have been detected in healthy adult blood (Katsura 1964; Gitlin et al. 1968; Boyer et al. 1975a, b; Wood et al. 1975).

In the present study, the mean HbA₁ weight in individual cells almost corresponded with HbA₁ weight in the average cell. But, the correlation coefficient between them was 0.546 ($p < 0.001$). The reason for this relatively low correlation is not clear. The diameter of red cells of 10 normal adults was determined on wet preparation by the method of Westerman et al. (1961). The mean diameter of red cells was not correlated with the mean HbA₁ weight in individual red cells, HbA₁ weight of the average red cell, and the total hemoglobin. This showed that the small red cell contained higher concentrated HbA₁ in comparison with the large red cell, and vice versa. This result almost agreed to that obtained by Ambs (1956), who found that the red cells with equal diameter showed various concentrations of total hemoglobin.

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References

- 1) Ambs, E. (1956) Durchmesser und Hämoglobingehalt der Erythrocyten. *Acta haemat. (Basel)*, **15**, 302-313.
- 2) Boyer, S.H., Belding, T.K., Margolet, L. & Noyes, A.N. (1975a) Fetal hemoglobin restriction to a few erythrocytes (F cells) in normal human adults. *Science*, **188**, 361-363.
- 3) Boyer, S.H., Belding, T.K., Margolet, L., Noyes, A.N., Burke, P.J. & Bell, W.R. (1975b) Variations in the frequency of fetal hemoglobin-bearing erythrocytes (F-cells) in well adults, pregnant women, and adult leukemics. *Johns Hopkins med. J.*, **137**, 105-115.
- 4) Daufi, L. & Rondell, P. (1969) Immunoprecipitation of diffusing antigens from single agar gel-suspended erythrocytes (Cytoimmunodiffusion). *Proc. Soc. exp. Biol. Med. (N.Y.)*, **131**, 1353-1356.

- 5) Gitlin, D., Sasaki, T. & Vuopio, P. (1968) Immunochemical quantitation of proteins in single cells. I. Carbon anhydrase B, β -chain hemoglobin and γ -chain hemoglobin in some normal and abnormal erythrocytes. *Blood*, **32**, 796-810.
 - 6) Headings, V., Bhattacharya, S., Shukla, S., Anyaibe, S., Easton, L., Calvert, A. & Scott, R. (1975) Identification of specific hemoglobins within individual erythrocytes. *Blood*, **45**, 263-271.
 - 7) Huisman, T.H.J., Martis, E.A. & Dozy, A.M. (1958) Chromatography of hemoglobin types on carboxymethylcellulose. *J. Lab. clin. Med.*, **52**, 312-327.
 - 8) Huisman, T.H.J. & Dozy, A.M. (1965) Studies on the heterogeneity of hemoglobin. IX. The use of tris (hydroxymethyl) aminomethane-HCl buffers in the anion-exchange chromatography of hemoglobins. *J. Chromatog.*, **19**, 160-169.
 - 9) Kamada, M. (1969) Red cells containing fetal hemoglobin in the blood of normal infants. *Yokohama med. Bull.*, **20**, 127-135.
 - 10) Katsura, S. (1964) A new serological method for the detection of fetal hemoglobin in the single erythrocyte. *Yokohama med. Bull.*, **15**, 117-126.
 - 11) Katsura, S., Taki, K. & Suzuki, K. (1976) Relationship between precipitate volume and antigen weight in immunodiffusion. *Tohoku J. exp. Med.*, **118**, 289-296.
 - 12) Mancini, G., Carbonara, A.O. & Heremans, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem.*, **2**, 235-254.
 - 13) Westerman, M.P., Pierce, L.E. & Jensen, W.N. (1961) A direct method for the quantitative measurement of red cell dimensions. *J. Lab. clin. Med.*, **57**, 819-824.
 - 14) Wood, W.G., Stamatoyannopoulos, G., Lim, G. & Nute, P.E. (1975) F-cells in the adult: Normal values and levels in individuals with hereditary and acquired elevations of HbF. *Blood*, **46**, 671-682.
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