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Age-dependent decay of cytochrome b_5 and cytochrome b_5 reductase in human erythrocytes

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Age-dependent decrease in cytochrome b_5 was observed in erythrocytes from both a normal person and a patient with hereditary methaemoglobinaemia without neurological symptoms. With aging, concentrations of cytochrome b_5 in erythrocytes from the patient were almost the same as those in the control. Age-dependent decrease in cytochrome b_5 reductase activity in the control erythrocytes was also shown; however, the reductase activity was very low in erythrocytes from the patient over the whole age range. Our studies show that methaemoglobin content of erythrocytes seems to be dependent on the content of cytochrome b_5 in the cells, both in the control subject and in the patient.

It has been reported that hereditary methaemoglobinaemia is due to the congenital deficiency of cytochrome b_5 reductase in erythrocytes or in cells of tissues (Hultquist & Passon, 1971; Sugita *et al.*, 1971; Leroux *et al.*, 1975; Tanishima *et al.*, 1980), and that NADPH–flavin reductase (NADPH–methaemoglobin reductase) (Yubisui *et al.*, 1979) is not involved in the disease, although added riboflavin accelerated the reduction of methaemoglobin in human erythrocytes (Matsuki *et al.*, 1978). Methaemoglobin reduction by NADH–cytochrome b_5 reductase (EC 1.6.2.2) needs a haemoprotein, cytochrome b_5, and the electron flow of the NADH–methaemoglobin reductase system is NADH–cytochrome b_5 reductase–cytochrome b_5–methaemoglobin. In erythrocytes from hereditary methaemoglobinaemic patients there has been no proof that cytochrome b_5 does not exhibit abnormality either in content or in ability to transfer electrons from cytochrome b_5 reductase to methaemoglobin. We first developed an assay method for cytochrome b_5 content of erythrocytes (Takeshita *et al.*, 1980) and applied it to the study of methaemoglobinaemia.

Several authors have reported the age-dependent decrease or the absence of ferrihaemoglobin–ferrocyanide-complex-reducing activity (Feig *et al.*, 1972; Vetrella *et al.*, 1971) or of NADH diaphorase (EC 1.6.4.3) activity (Keitt *et al.*, 1966) in erythrocytes from control subjects and from methaemoglobinaemic patients; however, there have been no reports about age-dependent changes in NADH–cytochrome b_5 reductase activity.

In the present paper age-dependent differences in cytochrome b_5 reductase and cytochrome b_5 in erythrocytes from a control subject and from a patient with hereditary methaemoglobinaemia are presented, and the role of cytochrome b_5 in the NADH-dependent methaemoglobin-reducing system is discussed.

Materials and methods

Blood from a normal control subject and from a hereditary methaemoglobinaemic patient was obtained from a volunteer and a patient in Toyoake with the agreement of each person. The diaphorase and NADH–cytochrome b_5 reductase activities in the patient's erythrocytes were extremely low, as reported by Tanishima *et al.* (1980). The blood samples (4ml) were freshly obtained with EDTA (dipotassium salt) as an anticoagulant, and were stored in an ice bath before use. Cytochrome b_5 was purified from human erythrocytes by the method of Passon *et al.* (1972). Cytochrome b_5 reductase was purified from human erythrocytes by the method of Yubisui & Takeshita (1980). Normal human blood was kindly supplied from The Japanese Red Cross Central Blood Center (Tokyo, Japan). All chemicals, of guaranteed reagent grade, were purchased commercially.

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Separation method

Erythrocytes were separated and washed with cold 0.9% NaCl (4°C; centrifugation at 2000*g* for 10min); plasma and buffy coats were removed three times. Washed erythrocytes were separated into four groups according to age by the method of Danon & Marikovsky (1964), which uses the differences in specific gravities of di-n-butyl phthalate/methyl phthalate mixtures. In this method 3vol. of the washed erythrocytes was layered over 1vol. of the densest mixture of the phthalate esters in a centrifuge tube. Specific gravities of mixtures used in this experiment were 1.098, 1.094, 1.090 and 1.086 at 20°C. The tube was then centrifuged in a swinging-bucket rotor at 12000*g* for 30min at 20°C. The upper fraction of the erythrocytes was suspended in 0.9% NaCl, layered on the mixture of sp.gr. 1.094 and centrifuged in the manner shown above. The lower fraction of erythrocytes was transferred to another tube and washed with 0.9% NaCl three times to remove the phthalate esters. All separation procedures were performed within 7h after the collection of blood. The separated erythrocytes were stored on ice before the next treatment. Cytochrome b_5 and cytochrome b_5 reductase in isolated erythrocytes were stable for at least 14 days and 25 days respectively at 4°C (Takeshita *et al.*, 1980; Tanishima *et al.*, 1980).

Assay methods

Methaemoglobin content was determined by the method of Evelyn & Malloy (1938).

Cytochrome b_5 reductase activity was assayed by the method of Yubisui & Takeshita (1980), by measuring the rate of increase of absorbance at 424nm (the absorbance maximum of the γ-band of reduced cytochrome b_5); ε_{mM} at 424nm was taken as 100. Each fraction of the erythrocytes separated into four groups according to age was haemolysed by adding 9vol. of cold distilled deionized water. The reaction mixture (2.0ml) contained 40μl of the haemolysate, 100μmol of potassium phosphate buffer, pH7.0, 1μmol of EDTA (disodium salt), 0.2μmol of NADH and 10nmol of cytochrome b_5. The reaction was initiated by the addition of NADH at 24°C.

Cytochrome b_5 content was determined by the method of Takeshita *et al.* (1980). To each fraction of the separated erythrocytes was added an equal volume of freshly prepared 0.2% $NaNO_2$ in 0.9% NaCl. The mixture was shaken gently and left for 20min at 25°C. The erythrocytes in each mixture were then washed six times with at least 10vol. of cold 0.9% NaCl and haemolysed by adding 19vol. of 25mM-sodium acetate buffer, pH5.6, at 4°C. The stroma fraction was removed by centrifugation at 10000*g* for 20min at 4°C, and the supernatant (met-haemolysate) was used for the determination of cytochrome b_5. The assay system contained 0.2ml of met-haemolysate, 100μmol of sodium acetate buffer, pH5.6, 30–40munits of NADH–cytochrome b_5 reductase (1 unit ≡ 1μmol of cytochrome b_5 reduced/min) and 300nmol of NADH in a total volume of 2.0ml. The reference cuvette contained the same constituents, except for reductase. Spectrophotometric measurements were carried out with a Cary model 17D double-beam spectrophotometer. Change in the absorbance at 576nm was recorded at 24°C. Standard assay mixture contained 0.01nmol of purified cytochrome b_5.

Results

Fig. 1 shows the age-dependent decay of cytochrome b_5 content in normal and the patient's erythrocytes. The cytochrome b_5 content in normal and the patient's erythrocytes decreased from young to old erythrocytes in the same manner, and exhibited no difference at the same age.

Fig. 2 shows the age-dependent decrease in cytochrome b_5 reductase activity. In normal erythrocytes the activities decreased with cell aging. In the patient's erythrocytes the activity of the youngest fraction was very low, and activity was undetectable in older fractions.

Fig. 3 shows the age-dependent increase in methaemoglobin content in erythrocytes. The methaemoglobin content in both the control and the patient's erythrocytes increased with cell age. The methaemoglobin content of the patient's erythrocytes was slightly lower on the whole than the amount of 17.8% found previously, because this patient has been treated with riboflavin (Hirano, 1980).

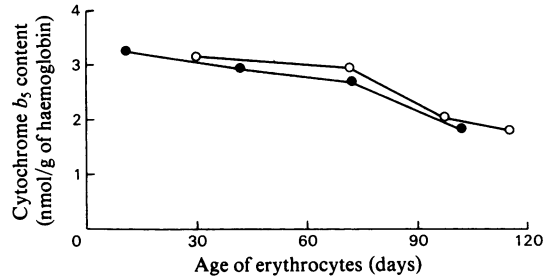

Fig. 1. *Age-dependent decay of cytochrome b_5 content in erythrocytes*

Each point indicates mean age of each fraction; 120 days was taken as the life span for human erythrocytes. Details of the separation of erythrocytes and assay conditions are given in the Materials and methods section. O, Control subject; ●, patient with hereditary methaemoglobinaemia.

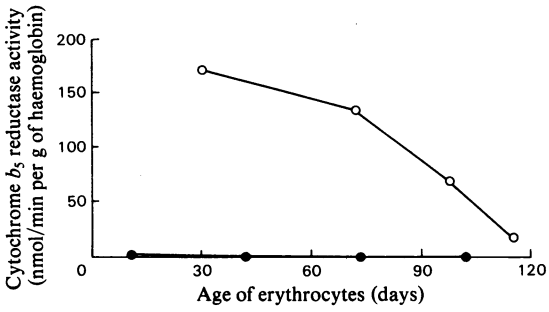

Fig. 2. *Age-dependent decrease of cytochrome b_5 reductase activity in erythrocytes*

Details of the assay condition are given in the Materials and methods section. O, Control subject; ●, patient with hereditary methaemoglobinaemia.

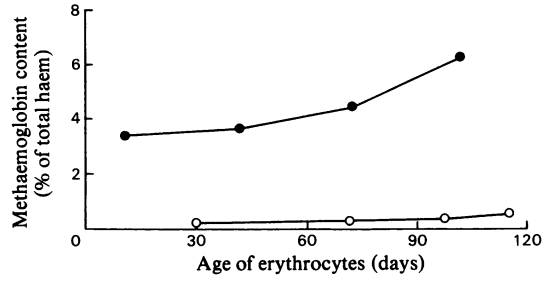

Fig. 3. *Age-dependent increases in methaemoglobin content in erythrocytes*

Methaemoglobin was determined from the absorbance at 630nm by the method of Evelyn & Malloy (1938). O, Control subject; ●, patient with hereditary methaemoglobinaemia.

Each point in Figs. 1–3 indicates mean age of each fraction; 120 days was taken as the life span for human erythrocytes.

Fig. 4 shows the correlation between the cytochrome b_5 content and the methaemoglobin content in each fraction of the patient's erythrocytes. The correlation coefficient was −0.99.

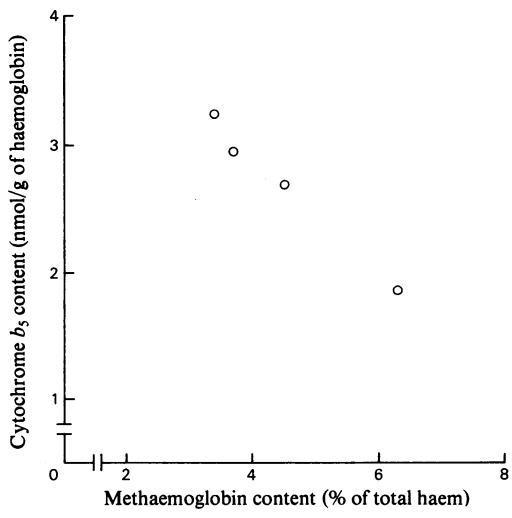

Fig. 4. *Correlation between cytochrome b_5 content and methaemoglobin content in erythrocytes of the patient with hereditary methaemoglobinaemia*

Data of Fig. 3 (methaemoglobin content) are plotted against the data of Fig. 1 (cytochrome b_5 content). The correlation coefficient was −0.99.

Discussion

Age-dependent decreases in enzyme activities or content of organic substances in human erythrocytes are known for such cases as glucose 6-phosphate dehydrogenase (Fornaini *et al.*, 1969), glutathione (Sass *et al.*, 1965) etc. (reviewed by Bunn, 1972). As shown in the Results section, NADH–cytochrome b_5 reductase activity and cytochrome b_5 content also decreased with aging in normal erythrocytes. The rate of decrease in the reductase activity was faster than that of the content of cytochrome b_5, and the methaemoglobin content was increasing. In this patient's erythrocytes, the abnormality is present in the activity of cytochrome b_5 reductase and not in the content of cytochrome b_5 in the erythrocytes.

The major system for methaemoglobin reduction in human erythrocytes has been considered to be the NADH–cytochrome b_5 reductase–cytochrome b_5–methaemoglobin system. In hereditary methaemoglobinaemia, deficiency of NADH–cytochrome b_5 reductase activity is known, and there has been no discrepancy between the occurrence of the disease and the deficiency of the enzyme activity. However, the enzyme cannot reduce methaemoglobin directly; it needs cytochrome b_5 as an intermediate for electron flow from NADH to methaemoglobin (Abe & Sugita, 1979). Moreover, there has been no report of a deficiency of cytochrome b_5, possibly because of the absence of an effective micro method for determination of cytochrome b_5 in tissues, especially in erythrocytes.

If the contents of cytochrome b_5 in erythrocytes from various people are assayed, methaemo-

globinaemia or other diseases induced by the deficiency or abnormality of cytochrome b_5 may be discovered.

As shown in the Results section, methaemoglobin content was correlated with cytochrome b_5 content for erythrocytes from both the control subject and the patient, and also with cytochrome b_5 reductase in erythrocytes from the control subject only. These results support the view that NADH–cytochrome b_5 reductase–cytochrome b_5–methaemoglobin must be the major system for methaemoglobin reduction in human erythrocytes, and indicate that methaemoglobin content may be dependent on the concentration of cytochrome b_5 and on the activity of cytochrome b_5 reductase in erythrocytes *in vivo*, as suggested by Sannes & Hultquist (1978) in their study *in vitro*. In erythrocytes from the patient, methaemoglobin concentration correlated well with cytochrome b_5 content in erythrocytes, as shown in Fig. 4. This result might indicate that cytochrome b_5 in erythrocytes is reduced not only by cytochrome b_5 reductase but also by other enzymes such as cytochrome *P*-450 reductase in ox liver (Enoch & Strittmatter, 1979).

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