TOCOPHEROL LEVEL IN HUMAN BLOOD CELLS

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Summary The study was undertaken on alpha-tocopherol levels, separated from other analogs, in the red blood cells (RBC) of cord blood, premature infants, healthy children and adults as well as in pregnant women, and compared with the plasma levels.

1. The majority of tocopherol found in the RBC was localized in the membranes.
2. Only alpha-tocopherol was found in the RBC, while alpha- and gamma-tocopherol were found in the plasma.
3. Alpha-tocopherol level in the RBC changes during development in parallel with that in the plasma. However, changes in the level in RBC were smaller than those of the plasma.
4. The ratio of alpha-tocopherol level in the RBC to the plasma was higher in the cord blood and premature infants than in the children and adults.
5. With regard to the cases with high plasma tocopherol levels, a different finding was obtained on the RBC level between pregnant women and adults with a large amount of tocopherol. In the latter, high RBC levels were observed as the plasma levels were elevated, while in spite of a high plasma tocopherol level, the lowest RBC levels were found in the pregnant women.

Keywords alpha-tocopherol, gamma-tocopherol, red blood cell, red blood cell membranes, plasma, HPLC, cord blood, children, adult, pregnant women

It is generally acknowledged that alpha-tocopherol operates to prevent acceleration of oxidative deterioration of biological membranes, where lipid peroxidation may be induced by active oxygen usually metabolized during the cell life (1, 2). Although recognition of alpha-tocopherol deficiency is clinically dependent on lowered plasma tocopherol levels and/or an increasing oxidative

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fragility of red blood cells (RBC) (3–6), it may be, however, more important to know the alpha-tocopherol level in the biological membranes with regard to functional impairment in the deficiency.

With technical advances in tocopherol assay, high-performance liquid chromatography (HPLC) was introduced (7,8), with which a highly sensitive separation and determination of alpha-tocopherol would be expected.

Biological membranes easily obtained from patients are from RBC, which are known to be destroyed in tocopherol deficiency by exposure to oxidative agents, such as hydrogen peroxide (3–6, 9, 10). Thus, in this paper, a study was undertaken on alpha-tocopherol levels, separated from other analogs, in RBC membranes in cord blood, premature infants, healthy children and adults, as well as in pregnant women, and compared with the plasma tocopherol levels.

MATERIALS AND METHODS

Cord blood was collected through the umbilical vein or artery after delivery in mature neonates with no complications, and maternal blood was drawn immediately before birth from healthy pregnant women. The blood specimens were obtained from premature infants at 4 to 38 days of age, who had no complications and whose birth weight was from 1,610 g to 2,500 g. The other blood specimens were obtained from eight healthy children, 1 to 14 years old (4 males and 4 females), as well as from healthy adults, 24 to 31 years old. The blood specimens in the children and adults were drawn after fasting in the morning.

Three healthy adults were intramuscularly administered with a single dose of 600 mg *dl*-alpha-tocopheryl acetate, and their blood was drawn at 3 days and 5 days after administration.

Preparation of RBC was carried out by the modified method of KAYDON (13, 14) as follows: Fresh blood, 5 ml, was transferred directly from a heparinized plastic syringe into the centrifuge tubes containing 0.1 ml of 1% NaEDTA, and were centrifuged at 2,000 rpm. After being separated from the plasma, the RBC layer was washed three times with 5 volumes of isotonic buffered saline² (pH 7.4) containing EDTA in the same concentration. Then, the final hematocrit of the RBC suspension was made up to about 50%, in which precise RBC counting and hematocrit measurement was carried out.

Preparation of RBC membranes was performed by the method of DODGE (12).

The procedure of extraction of tocopherol and of HPLC by a minor modification of ABE's method (7) was as follows: 0.5 ml of plasma was placed into a glass-stoppered 12-ml centrifuge tube, into which 0.5 ml of distilled water and 1 ml of tocol solution (2 µg/ml ethanol) were added. After vigorous shaking, 5 ml of *n*-hexane was added and was shaken again for 1 min. The mixture was centrifuged at 1,000 rpm for 5 min, and 4 ml of hexane layer was evaporated under nitrogen gas

² The buffer solution was prepared in a mixture of the same volume of 0.15 M phosphate buffer and *n*-saline.

flow at 40°C. The residue was dissolved in 50 μ l of hexane, in which 20 μ l was injected into the HPLC column.

With regard to alpha-tocopherol determination in RBC or RBC membranes, 0.5 ml of an RBC suspension or RBC membrane suspension was placed into the centrifuge tube where 1 ml of 6% pyrogallol solution in ethanol and the same volume of tocol solution as described above and 0.2 ml of 60% KOH were added, and the tube was incubated at 70°C for 30 min for saponification. After cooling, 2.5 ml of water and 5 ml of hexane were added to the tube, then extraction into the hexane layer was performed in the same manner as the preparation of plasma tocopherol.

The instruments used in this study were as follows: HPLC: FLC-350 (Japan Spectroscopic Co., Ltd.), Column: JASKOPACK-WC-03-500 (Partition, 2.3 mm *i.d.* $\times$ 500 mm), Detector: Fluorospectrophotometer, JASKO FP-4 type (Ex. 298 nm, Em. 325 nm) or Spectrophotometer, JASKO UVIDEK -2 type (295 nm). Solvent: Isopropylether/*n*-hexane = 0.5/99.5. Flow rate; 1.0 ml/min.

Retention time was 2.2 min for alpha-, 3.8 min for beta-, 4.6 min for gamma-, 6.9 min for delta-tocopherol and 10.0 min for tocol, respectively.

RESULTS

Pattern of tocopherols detected by HPLC analysis are shown in Fig. 1. Alpha- and gamma-tocopherol were detected in the 0.2 ml of plasma, while only the alpha form was detected in the RBC as well as in the RBC membranes corresponding to the 0.25 ml of packed cells.

Table 1 shows the recovery test of alpha-tocopherol in the specimens. A satisfactory result was obtained in the recovery of alpha-tocopherol added to the plasma and RBC.

Alpha-tocopherol levels analyzed from the total RBC were compared with those from membranes prepared by the method of Dodge. As shown in Table 2, similar tocopherol levels were observed in both. This finding indicates that the majority of tocopherol is localized in the membranes. Thus, further examination was carried out in the determination from total RBC, owing to the technical complexity of membrane preparation.

The four cord blood specimens were separately collected through the umbilical vein and artery, and the alpha-tocopherol levels were compared. As shown in Table 3, similar results were obtained in the plasma and in the RBC. Hence, other cord blood was drawn only through the umbilical vein.

Table 4 shows the level of plasma and RBC in the cord blood as compared with the mother's. It was surprising that no significant difference was observed in the two levels of RBC, while in the plasma the mother's level was higher than in the cord blood.

The level of plasma and RBC in premature infants, healthy children and adults are shown in Tables 5, 6 and 7, respectively. From the results, changes in the

Fig. 1. Patterns of tocopherols isolated by HPLC in the standard, plasma and RBC (or their membranes).

Retention time of tocopherols: α -Toc, 2.2 min; β -Toc, 3.8 min; γ -Toc, 4.6 min; δ -Toc, 6.9 min; Tocol, 10.0 min.

Table 1. Result of recovery test in the assay procedure for alpha-tocopherol in the plasma and RBC.

Specimen	Alpha-tocopherol level (μ g)			Recovery (%)
	Added	Theoretical	Determined	
(Plasma)				
8.65	5.00	13.65	13.60	99.6
8.65	10.00	18.65	18.88	102.3
9.97	5.00	14.97	15.21	104.4
9.97	10.00	19.97	20.19	102.2
(RBC)				
1.76	5.00	6.76	6.39	92.6
1.75	10.00	11.76	12.07	103.2
1.30	10.00	11.30	11.57	102.7
1.23	5.00	6.23	6.29	101.2

Table 2. Alpha-tocopherol level in RBC membranes as compared with that in total RBC.

No.	RBC	RBC membrane
	(mg/dl · RBC) (as packed cell)	(mg/membranes in the same volume of RBC)
1	0.25	0.21
2	0.15	0.19
3	0.15	0.19
4	0.26	0.20
5	0.36	0.34
mean ± SD	0.23 ± 0.08	0.23 ± 0.06

RBC membranes prepared by the method of Dodge were saponified and lipids were eluted with *n*-hexane by the same procedure for RBC assay.

Table 3. Comparison of alpha-tocopherol level between venous and arterial blood drawn from umbilical cords.

No.	Venous blood			Arterial blood		
	Plasma	RBC		Plasma	RBC	
	(mg/dl)	(mg/dl · RBC) (as packed cell)	(ng/10 ⁸ · RBC)	(mg/dl)	(mg/dl · RBC) (as packed cell)	(ng/10 ⁸ · RBC)
1	0.28	0.16	18.4	0.29	0.15	16.4
2	0.40	0.15	15.4	0.47	0.18	18.6
3	0.40	0.12	11.9	0.40	0.16	16.9
4	0.43	0.12	12.0	0.43	0.12	12.2
mean ± SD	0.38 ± 0.07	0.14 ± 0.02	14.4 ± 3.1	0.40 ± 0.08	0.15 ± 0.03	16.0 ± 2.7

RBC counting was carried out from a 50% suspension made up after washing.

levels in the plasma and RBC during development are shown in Fig. 2. Alpha-tocopherol levels in the RBC changed in parallel with those in the plasma during development. However, changes in the level of RBC were smaller than those of the plasma during development. Accordingly, the ratio of the level in RBC to that in plasma was observed in the cord blood, premature infants, children and adults, and was compared with that of pregnant women, as shown in Fig. 3. The ratio was higher in the cord blood and premature infants than in the children and adults. The lowest occurred in the pregnant women. This finding suggests that plasma tocopherol level don't directly reflect the level in RBC membranes. Then, whether

Table 4. Alpha-tocopherol level in RBC of maternal and cord blood as compared with that in plasma.

No.	Maternal blood			Cord blood		
	Plasma	RBC		Plasma	RBC	
	(mg/dl)	(mg/dl · RBC) (as packed cell)	(ng/10 ⁸ · RBC)	(mg/dl)	(mg/dl · RBC) (as packed cell)	(ng/10 ⁸ · RBC)
1	1.70	0.17	9.2	0.28	0.16	18.4
2	2.11	0.17	15.6	0.40	0.15	15.4
3	2.30	0.18	14.7	0.40	0.12	11.9
4	2.25	0.14	11.9	0.43	0.12	12.0
5	1.16	0.18	13.3	0.46	0.21	21.2
6	2.99	0.20	16.1	0.40	0.19	20.6
7	1.56	0.22	21.8	0.39	0.19	20.2
mean ± SD	2.01 ± 0.60	0.18 ± 0.03	14.7 ± 3.9	0.39 ± 0.06	0.16 ± 0.04	17.1 ± 4.0

The correlation is shown between simultaneous maternal and cord blood.

Table 5. Alpha-tocopherol levels in RBC and plasma of premature infants.

No.	Brith weight	Age	Present weight	Plasma	RBC	
	(g)	(days)	(g)	(mg/dl)	(mg/dl · RBC) (as packed cell)	(ng/10 ⁸ · RBC)
1	1,610	5	1,520	0.64	0.14	14.7
2	1,880	4	1,880	0.28	0.08	9.3
3	2,170	16	2,350	0.92	0.24	24.6
4	2,200	9	2,060	0.32	0.14	15.0
5	2,200	21	2,700	0.54	0.20	20.2
6	2,220	38	2,880	0.31	0.17	15.5
7	2,240	7	2,080	0.33	0.10	11.3
8	2,240	36	2,500	0.32	0.14	15.0
9	2,460	16	2,640	0.63	0.22	23.0
10	2,500	12	2,660	0.49	0.25	24.8
mean ± SD				0.48 ± 0.21	0.17 ± 0.06	17.3 ± 5.5

Blood was drawn immediately before feeding (3 hours' fasting).

alpha-tocopherol level in the RBC is influenced as the plasma level is elevated by an administration of a large amount of tocopherol, was examined.

Administration of tocopheryl acetate was carried out under the conditions described above. Since a small amount of tocopheryl acetate is known to remain in

Table 6. Alpha-tocopherol levels in RBC and plasma of the children.

No.	Age (Yrs.)	Sex	Plasma (mg/dl)	RBC	
				(mg/dl · RBC) (as packed cell)	(ng/10 ⁸ · RBC)
1	1	M	0.96	0.35	26.7
2	6	M	0.84	0.19	15.4
3	9	M	0.97	0.28	22.1
4	12	M	1.05	0.24	23.9
5	3	F	1.05	0.22	18.1
6	7	F	0.82	0.15	15.2
7	12	F	1.01	0.25	26.1
8	14	F	0.53	0.15	15.6
mean ± SD			0.90 ± 0.17	0.23 ± 0.07	20.4 ± 4.9

Blood was drawn after overnight fasting.

Table 7. Alpha-tocopherol levels in RBC and plasma of healthy adults.

No.	Plasma (mg/dl)	RBC	
		(mg/dl) (as packed cell)	(ng/10 ⁸ · RBC)
1	0.87	0.24	22.7
2	1.32	0.15	13.4
3	0.76	0.17	15.3
4	0.60	0.17	15.9
5	0.99	0.24	21.0
6	0.58	0.18	17.6
7	0.83	0.17	15.9
8	0.73	0.19	13.4
9	0.75	0.18	15.2
10	0.68	0.19	18.8
11	0.82	0.20	16.3
12	1.20	0.31	30.8
mean ± SD		0.20 ± 0.04	18.0 ± 4.9

Blood was drawn after overnight fasting.

the plasma for more than 24 hours after intramuscular administration (21), the assay of tocopherol in the plasma was performed by the same method with saponification as for RBC assay. Results are shown in Table 8. With increased plasma tocopherol levels after loading, the levels in RBC were also elevated, while

Fig. 2. Changes in alpha-tocopherol levels in plasma and RBC during development. Mean values $\pm$ SD shown in the figure result from the values in Tables 4 to 7.

the ratios of alpha-tocopherol in RBC to plasma were nearly constant even when the blood tocopherol level was elevated.

DISCUSSION

There have been few investigations into alpha-tocopherol levels in RBC owing to technical difficulties of the assay. In previous reports (11, 13, 14), the assay was carried out using TLC and/or GLC techniques, and in association, further correction, calculated from the recovery of isotope-labeled tocopherol added at the beginning of procedure, was necessary. The method introducing HPLC, as devised by ABE(7) and ISHIBASHI(8), was used in our study; this gave a simple

Fig. 3. Changes in the ratios of alpha-tocopherol in RBC to that in plasma during development, and compared with the ratios in pregnant women. Mean values $\pm$ SD shown in the figure were calculated from the values in Tables 4 to 7. Tocopherol levels of RBC used for calculation were the values per packed cell.

Table 8. Changes in alpha-tocopherol level of RBC and plasma after the intramuscular administration of a large amount of *dl*-alpha-tocopheryl acetate.

Case	Age	Sex		Alpha-tocopherol		Tocopherol ratio of RBC to plasma
				Plasma (μ g/dl)	RBC (μ g/dl)	
1	32	M	Before	651	180	27.6
			After (3 days)	1,420	419	29.5
			(5 days)	981	251	25.5
2	29	M	Before	605	143	23.6
			After (3 days)	1,308	419	32.0
			(5 days)	1,052	286	27.1
3	29	F	Before	891	263	29.5
			After (3 days)	1,851	523	28.2
			(5 days)	1,255	365	29.0

Intramuscular administration with a single dose of 600 mg of *dl*-alpha-tocopheryl acetate was performed. Blood was drawn at 3 days and 5 days after the administration. The assay for plasma was carried out with the treatment of saponification, since a small amount of the tocopheryl acetate administered may remain in the plasma.

procedure, and also high sensitivity and reproducibility for tocopherol assay in the RBC, in which 10 or 15% of tocopherol in the whole blood was detected. Thus,

correction based on recovery was unnecessary, as almost 100% recovery was directly obtained.

With regard to plasma tocopherol, we obtained similar results to those reported by ABE (15); more than 85% of the total tocopherol was the alpha form, but only a small amount of the gamma form was detected, while no beta- or delta-tocopherol was detected.

With regard to RBC tocopherol, only the alpha form was detected in our study, while CHAW (16) and ISHIBASHI (8) detected the alpha form, which accounted for the majority of the total tocopherol, and a much smaller amount of the gamma form. Since the alpha-tocopherol level in the RBC in our study was similar to that reported by Ishibashi, no detection of the gamma form is possibly due to too small an amount of RBC being used for our assay.

The finding that alpha-tocopherol is localized only in the membranes of RBC, is in agreement with CHOW (16) and SILBER (14). CHOW used direct measurement of RBC membranes, while SILBER used isotope-labeled tocopherol.

Investigation of tocopherol in human RBC was carried out by BIERI (17) and KAYDON (13, 14). The level given by Bieri is coincident with ours, while Kaydon's is slightly lower. The difference in the levels was not significant.

ISHIBASHI (8) reported that the ratio of tocopherol in RBC to plasma differs between animal species, but is relatively constant within the same species, even if the tocopherol level is increased by supplement. Ishibashi's results suggest that the plasma tocopherol level reflects the level in membranes. As shown in Table 8, when the tocopherol level in plasma is elevated by loading, a rise of its level in the RBC reflected the plasma level. This finding is in agreement with Ishibashi's view. In contrast, the ratio differed with human development, as well as in the pregnant women in our study. The difference of the ratios during development resulted from the fact that the rise of the level in RBC was much smaller than that in the plasma, *i.e.*, RBC membranes in the cord blood and premature infant with the lowest plasma level maintained their tocopherol level. Such a relationship between the plasma and RBC tocopherol levels has been indicated in the cases with abetalipoproteinemia as reported by BIERI (11).

The finding of a low RBC level in spite of a high plasma level in pregnant women, while with loading a large amount of tocopherol there was a high level in the plasma as well as in RBC, could not adequately be interpreted. BIERI (17, 18) proposed that a distribution of tocopherol in the components of the body is due to a mass action effect of the lipids, *e.g.* it may be distributed simply in the lipid mass of body components such as adipose tissues, plasma lipids, lipoproteins and membrane lipids, *etc.* The hypothesis is based on the finding (18) that in obese rats with hyperlipemia, although the plasma tocopherol was elevated, the tissue tocopherol content was lower than in normolipemic, nonobese rats. It also derives from the additional finding (17) that RBC content of tocopherol decreased as plasma lipid increased when the distribution of isotope-labeled tocopherol in the *in vitro* reaction system between the plasma, which was experimentally made on hypo-

and hyperlipemia, and RBC was examined. The recent report by MACHLIN (19) may reflect Bieri's view, that in a guinea pig alternately fed a diet with and without vitamin E histological evidence of a vitamin E deficiency (myopathy) appeared at the time when adipose tocopherol stores were not significantly different from the initial values. These findings suggest that the various lipid pools in the body act as a "sink" for tocopherol and can drastically affect how the various nonlipid tissues take up the vitamin. Thus, tocopherol taken up into a large lipid mass may lose the function for transport to other components and for exchange between the lipid phases.

From this standpoint, it thus appears that in order to interpret how tocopherol levels in plasma relate to the membrane status of vitamin E, it is necessary to take into consideration the degree of adiposity of the body components, as emphasized by Bieri.

With regard to maternal lipid metabolism, it is well known that lipid stores in maternal adipose tissues increase over the first two-thirds of gestation, while toward the end of pregnancy lipolysis in adipose tissue increases and circulating FFA concentration rises (20). Thus, the point arising as to whether uptake of tocopherol occurs more predominantly into the circulating lipids than into the RBC membranes in the pregnant women, remains to be studied.

From the standpoint regarding the membrane action of tocopherol, it may be of dubious value to take into consideration that the biological importance of tocopherol in the membranes is regulated and maintained only by its distribution among the lipid phases in the body components. Further study is required to elucidate this problem.

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