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Comparison of Vitamin E Levels in Human Plasma, Red Blood Cells, and Platelets Following Varying Intakes of Vitamin E

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Summary Plasma, red blood cells (RBC), and platelets from young female subjects on varying intakes of vitamin E (VE) as butter or margarine (rich in VE) were analyzed for tocopherols to estimate which blood components most closely followed changes in the dietary intake. The VE nutritional status of the subjects at the beginning of the study was marginally deficient from the normal range of RBC α-tocopherol. No significant changes in the plasma α-tocopherol concentrations were observed, even though the VE intake had increased. The α-tocopherol level in RBC, however, increased with an increase in VE intake; and that in platelets, to a lesser extent. The plasma lipid peroxide levels were lowered with increased VE intake, showing an inverse change to the α-tocopherol levels in red blood cells and platelets. Consequently, changes in α-tocopherol levels in RBC closely followed varying dietary intakes of VE when compared with data from platelets and plasma.

Key Words: vitamin E, plasma, red blood cell, platelet, lipid peroxide

As a naturally occurring antioxidant, vitamin E (VE) acts to protect organisms against the harmful attacks of oxidants. Therefore, it is important to assess the nutritional status of VE to evaluate antioxygenic capacity of protection against oxidant attacks. VE nutritional status in humans has customarily been estimated by measurement of the plasma tocopherol concentration because of its simplicity and convenience. However, the close correlation between plasma tocopherol and

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plasma lipid levels raised serious doubts about the validity of its estimation [1–3], and the ratio of plasma tocopherol to plasma lipid levels is proposed as a valid criterion for the assessment of VE status [1]. We also pointed out the validity of the ratio in connection with the change in serum lipid peroxide level in an elderly population [4]. Moreover, it is suggested that the ratio of plasma VE to plasma polyunsaturated fatty acid concentrations may be useful to evaluate lung VE level when it is expressed in terms of polyunsaturated fatty acid level [5]. However, since VE does not function in plasma but rather at the cellular level, localization of the vitamin in membranes should be considered in the assessment of VE nutritional status. In research directed ultimately toward human nutrition, feasible cell systems for estimating tissue VE status are, for all practical purposes, limited to those in the blood such as platelets and red blood cells (RBC). And the VE level in platelets [6–8] or RBC [9–12] is proposed as a good measure for VE status, although it is still controversial as to which is the better measure.

It is generally accepted that a principal function of VE is to prevent peroxidation of unsaturated fatty acids in membrane phospholipids. Lipid peroxidation is a destructive process that can alter membrane function and stability. Increased amounts of dietary polyunsaturated fatty acids thus increase the requirement for VE for prevention of lipid peroxidation [13–20]. Accordingly, the determination of plasma lipid peroxide level as well as the RBC hemolysis test [21–23] is also considered to be useful for the assessment of VE nutritional status.

In the present study, diets differing remarkably in VE and polyunsaturated fatty acid contents were supplied to young female subjects, and the VE levels in plasma, RBC, and platelets, and also the plasma lipid peroxide level, were analyzed to see which blood components reflected the changes in VE intake.

SUBJECTS AND METHODS

Subjects and meals. Twelve healthy Japanese female university students volunteered as subjects for the study. All subjects were assigned to the first 4-day period of the study and then randomly divided into two groups of 6 subjects each for its second 4-day period. Four kinds of daily menu for the 4-day period were set and repeated in the same sequence in the first and second periods of the study except daily intake of 90 g of butter or margarine was added in the second period. The daily intake of nutrients, representing 1,400 kcal of energy, included 70 g of protein and 10 g of sodium chloride. Other nutrients were supplied according to the dietary allowances for female Japanese. In addition to these daily nutrients, half of the subjects ingested 90 g of commercial butter (670.5 kcal/day; no tocopherol added) and half, margarine (683.1 kcal/day; *d*-α-tocopherol added; rich in linoleic acid, 65%) with dishes every day in the second period. Nutrient intakes were calculated from a food composition table [24]. Participants were not taking prescription drugs or other vitamin supplements during the study. Peroxide values of butter and margarine used were 0 and 0.35 (meq/kg), respectively; and thiobar-

bituric acid (TBA) values, 2.5 and 3.2 (nmol malondialdehyde/g).

Analysis of VE in diet. VE (tocopherols) contents in the diets were analyzed as follows: Dishes of food identical to those consumed were mixed together in a mechanical mixer on a daily basis and then made up to a constant volume. An aliquot was extracted with chloroform-methanol (2 : 1, v/v) and then benzene. The extracts soluble in *n*-hexane, to which 2,2,5,7,8-pentamethyl-6-hydroxychroman [25] was added as an internal standard, were subjected to high-performance liquid chromatography (HPLC) on an apparatus equipped with a fluorometer [4]. The operating conditions for HPLC were as follows: instruments, Hitachi L-6000 type liquid chromatograph (Hitachi Ltd., Katsuta, Japan); detector, Hitachi F-1100 spectrofluorometer (Hitachi Ltd.) (Ex 298 nm, Em 325 nm); column, stainless steel tube (4.6 × 150 mm) packed with Zorbax ODS (Lockland Technology Inc., Westchester); mobile phase, 99 : 1 mixture of *n*-hexane and isopropyl alcohol; flow rate, 0.5 ml/min. The relative biological activities for *d*-α-, *d*-β-, *d*-γ-, and *d*-δ-tocopherols were taken as 100 : 25 : 5 : 0.1 in the calculation [26], and daily intakes of VE were expressed in terms of *d*-α-tocopherol equivalents.

Preparation of plasma, RBC, and platelets. After overnight fasting, blood samples were drawn at the beginning (pre-period) and at the end of each of the two test periods (first period and second period). Procedures for blood collection and cell separation were carried out using plastic ware. Four milliliters of venous blood containing 0.2 ml of a 2% solution of ethylenediaminetetraacetic acid (disodium salt) as anticoagulant were centrifuged at 150 × *g* for 10 min at 4°C to separate platelet-rich plasma (PRP). The PRP was transferred to another centrifuge tube and the buffy coat/RBC layer recentrifuged at 2,700 × *g* for 10 min at 4°C. The plasma was separated and the lower layer used to isolate RBC. Platelets were separated from the PRP by centrifugation at 800 × *g* for 10 min at 4°C, washed twice with physiological saline, diluted with the saline, and then freeze-thawed twice to give a homogenous suspension with a protein concentration [27] of 1–2 mg/ml. For RBC, the buffy coat was removed and the remaining cells were washed three times with physiological saline, and diluted 1 : 1 with the saline to prepare an approximately 50% suspension of RBC. The hematocrit of the diluted RBC was measured with a microcapillary centrifuge and used to calculate RBC volumes. The plasma separated from a recentrifugation of the buffy coat/RBC layer as above described and from the 800 × *g* centrifugation of PRP were combined for analysis. Plasma, RBC, and platelet samples were stored at −80°C until analyzed except for the analysis of plasma lipid peroxides, which was performed immediately after plasma preparation.

VE analysis in plasma, RBC, and platelets. Tocopherols in plasma [4] and RBC [12] were analyzed by HPLC. Analysis of tocopherols in platelets was also according to the method devised for determination of RBC tocopherols [12]. 2,2,5,7,8-pentamethyl-6-hydroxychroman [25] was used as an internal standard in place of all-rac-tocol. The operating conditions for HPLC were the same as those described above for the dietary VE analysis. α- and γ-tocopherols accounted for

nearly all of the VE compounds in plasma, RBC, and platelets. The values of tocopherols in RBC were corrected by the hematocrit values of each sample.

Miscellaneous analyses. Plasma total cholesterol (Determiner TC 555, Kyowa Medex Co. Ltd., Tokyo), phospholipids (Determiner PL, Kyowa Medex Co. Ltd.), and triglycerides (Nescauto TG-A, Nihonshoji Co. Ltd., Osaka) were determined by use of standard clinical chemistry kits available commercially. Total lipids were estimated by summation of the three major lipids. Plasma lipid peroxide level was measured by TBA [28] and water-soluble fluorescent pigment [29] methods.

The study protocol was approved by the Institute Ethnic Committee and the studies were pursued after informed consent had been obtained from the subjects.

Statistical analysis. After confirming the homogeneity of variance of data of the groups examined by employing the Bartlett test, we assessed statistical significance of difference between mean values by analysis of variance (ANOVA) coupled with Duncan's multiple-range test [30] at the 5% level of significance.

RESULTS

Daily VE intakes

The daily intake of VE from food dishes as *d*-α-tocopherol equivalents for each of the four days in the first period was 3.0, 9.5, 11.5, and 2.7 mg/day, respectively, with a mean value of 6.7 mg/day; and in the second period, it was 8.8, 15.4, 17.4, and 8.6 mg/day with a mean value of 12.6 mg/day in the butter group and 48.9, 55.4, 57.4, and 48.6 mg/day with a mean value of 52.6 mg/day in the margarine group. The daily intakes of *d*-α-tocopherol from butter and margarine were 5.9 and 45.8 mg/day, respectively. Almost all the VE in butter and margarine was *d*-α-tocopherol.

Variations of plasma VE levels

Table 1 represents the variations of VE levels in plasma, RBC, and platelets. Plasma α-tocopherol levels did not change significantly during the study and thus did not reflect the increase in VE intake concomitant with butter or margarine ingestion. The plasma γ-tocopherol level, however, decreased significantly following the high intake of VE attended with margarine ingestion. Butter ingestion slightly increased the level of plasma total lipids and margarine decreased the level, resulting in a significant difference between butter and margarine groups. Accordingly, the plasma α-tocopherol to total lipid ratio declined in the butter group and rose in the margarine group. A similar tendency was observed for the ratio of γ-tocopherol to total lipid.

Variations of RBC VE levels

The α-tocopherol level of RBC of the subjects, judging from the normal range [12], was rather low at the beginning of this study (pre-period). Consequently, the

Table 1. Variations of plasma, red blood cell and platelet vitamin E levels during test periods.

	Pre-period	First period	Second period	
			Butter	Margarine
Number of subjects	12	12	6	6
Plasma α-tocopherol (μg/dl)	845.2±166.9[a]	834.2±95.9[a]	827.8±156.9[a]	808.1±57.8[a]
Plasma γ-tocopherol (μg/dl)	42.7±12.1[a,b]	54.1±15.4[a]	50.7±16.1[a]	32.3±10.8[b]
Plasma total lipids (mg/dl)	385.2±50.2[a,b]	384.3±52.5[a,b]	429.0±61.2[a]	348.2±32.3[b]
Plasma α-tocopherol/total lipid (mg/g)	2.18±0.25[a,b]	2.20±0.36[a,b]	1.92±0.14[a]	2.33±0.17[b]
Plasma γ-tocopherol/total lipid (mg/g)	0.11±0.03[a,b]	0.14±0.05[a]	0.12±0.03[a,b]	0.09±0.02[b]
Red blood cell α-tocopherol (μg/dl packed cells)	95.8±19.8[a]	184.1±38.9[b]	196.4±57.5[b]	295.1±61.9[c]
Red blood cell γ-tocopherol (μg/dl packed cells)	8.5±2.9[a]	18.4±4.2[b]	14.9±4.4[b]	16.1±3.6[b]
Platelet α-tocopherol (ng/mg protein)	271.7±57.9[a]	322.1±39.8[b]	301.1±47.3[a,b]	352.9±22.5[b]
Platelet γ-tocopherol (ng/mg protein)	22.7±6.2[a]	23.7±4.0[a]	25.7±4.6[a]	27.1±3.0[a]

All data are given as means±SD. Means within the same row that are not followed by a common superscript letter are significantly different ($p<0.05$).

level significantly augmented even in the first period with a mean intake of 6.7 mg/day as α-tocopherol equivalents. The increase in VE intake concomitant with margarine ingestion in the second period further elevated the level linearly. However, in the butter group the α-tocopherol level did not increase significantly in spite of an almost twofold increase in the VE intake over that of the first period. The γ-tocopherol level of RBC similarly increased in the first period; but no definite changes were noticed in the second period, even in the margarine group.

Variations in platelet VE levels

The α-tocopherol levels of platelets varied very similarly with those of RBC during the study but to a lesser extent. No significant changes were recognized in the γ-tocopherol levels of platelets during the study.

Variations in plasma lipid peroxide levels

Table 2 indicates the variations in plasma lipid peroxide levels as measured by TBA value and water-soluble fluorescent substance level. The TBA value of the first period was significantly lower as compared with that of the pre-period, showing an inverse relation to the α-tocopherol levels in RBC and platelets. In the second period the TBA values further decreased as the dietary intake of VE increased following butter or margarine ingestion, where the TBA value of the margarine group tended to be lower than that of the butter group; although there was no statistical difference between the two groups. Plasma levels of water-soluble fluorescent substances also changed similarly as the TBA values but to a lesser

Table 2. Variations of TBA value and water-soluble fluorescent substance level in plasma during test period.

	Pre-period	First period	Second period	
			Butter	Margarine
Number of subjects	12	12	6	6
TBA value (nmol MDA/ml)	4.6 ± 0.9^{a}	3.9 ± 0.7^{b}	2.5 ± 0.3^{c}	2.0 ± 0.2^{c}
Water-soluble fluorescent substances (relative fluorescence intensity)	16.2 ± 2.8^{a}	15.8 ± 3.2^{a}	15.0 ± 2.7^{a}	14.6 ± 3.0^{a}

All data are given as means$\pm$SD. Means within the same row that are not followed by a common superscript letter are significantly different ($p<0.05$).

extent with no significant differences.

DISCUSSION

In the present study we selected at random the female university students as subjects, but their VE nutritional status at the beginning of the study (pre-period) was marginally deficient judging from the lower limit of the normal range of RBC α-tocopherol (115 μg/dl packed cells) [12]; although it was acceptable status from the plasma α-tocopherol concentration [4] and/or plasma α-tocopherol to total lipid ratio [1]. However, this nutritional status made clear the susceptibility of blood components to a variation in dietary VE intake and a resultant change in VE nutritional status, where the body reflects most remarkably the change in VE status at the effective sites of the body when VE is depleted or marginally deficient. There is a body of evidence to show this as discussed below.

First, no significant changes in the plasma α-tocopherol concentrations were observed during the study, although the VE intake concomitant with butter or margarine ingestion increased. The VE nutritional status of the subjects was marginally deficient at the beginning of the study, as already described, where the storage of α-tocopherol in tissues including RBC was insufficient, leading to a lack of response of plasma α-tocopherol concentrations to the changes in plasma total lipid concentrations following butter or margarine ingestion. However, the elevated intake of α-tocopherol attended with margarine ingestion depressed significantly the plasma γ-tocopherol concentration in agreement with previous observations [31, 32]. Thus, in spite of no change in plasma α-tocopherol concentration of the subjects, the elevated bodily α-tocopherol was preferentially retained over the short duration of the trial especially at the effective sites of the body including RBC and platelets, as discussed below. Handelman *et al.* [32] showed that the depression in plasma γ-tocopherol evidently could occur during large dietary α-tocopherol intake even without a large absolute increase in α-tocopherol in the plasma, and thus suggested that a decrease particularly in the ratio of γ- to α-tocopherol might provide a much more reliable index of compliance to α-tocopherol supplementation. The ratios of γ- to α-tocopherol calculated from the mean values shown in Table 1 were 0.051, 0.065, 0.061, and 0.040

for pre-period, first period, and butter and margarine groups of the second period, respectively. When plasma α-tocopherol concentrations were expressed in terms of a ratio of plasma α-tocopherol to total lipids, the ratio increased in the margarine group, reflecting a decrease in the plasma total lipid concentration, and vice versa in the butter group.

Second, the α-tocopherol levels of both RBC and platelets significantly increased even in the first period with lower intake of α-tocopherol, and further elevated linearly, particularly in the RBC of the margarine group in the second period. Relative merits of the RBC and platelets to reflect changes in VE intake were calculated by the sensitivity concept [8], i.e., rate of change of tocopherol levels with dose divided by the standard deviation. The sensitivity of platelets relative to RBC for measuring VE intake was 0.76 in the margarine group in the second period and 0.56 in the first period. Accordingly, changes in α-tocopherol levels in RBC more closely followed varying dietary intakes of VE when compared with data from platelets. As it is reported that a linear relationship is found between platelet tocopherol content and the dosage of *dl*-α-tocopherol consumed until the dosage reached 1,800 IU/day (1,208 mg/day as *d*-α-tocopherol equivalents) in human subjects [33], the lower sensitivity of platelets in the margarine group compared with that of RBC is not due to a saturation of platelets with α-tocopherol. In addition, there are also data indicating that RBC are not saturated with α-tocopherol up to four times the normal plasma level of α-tocopherol [34]. Although the subjects in the butter group ingested almost twofold higher VE than in the first period, no further increases were noticed in both the RBC and platelets. This level of increase in VE intake does not appear to be reflected by the α-tocopherol levels of RBC and platelets.

In the margarine group VE intake increased concomitant with margarine ingestion, but the plasma α-tocopherol concentration did not change and the α-tocopherol levels of RBC and platelets increased. These results seem to mean that, in an inadequate VE nutritional status, ingested VE is preferentially utilized at the effective sites of biomembranes, leading to increases in tissue tocopherol including RBC and platelets available for biological function in the biomembrane but not to an increase in plasma tocopherol concentration. Moreover, the degree of increase in α-tocopherol following the margarine ingestion was higher in RBC than in platelets. This, from a different viewpoint, appears to indicate that a decrease in VE while very low intake of VE continues for a prolonged period is faster in RBC than in platelets, presuming that the priority for VE in RBC is inferior to that in platelets. This concept is supported by the results to show that the loss of VE in the process leading to VE deficiency is faster in RBC than in platelets in rats [35].

Third, the plasma lipid peroxide levels as measured by TBA value and water-soluble fluorescent substance level changed inversely with the changes in α-tocopherol levels in RBC and platelets. When the lipid peroxide levels of butter and margarine groups were compared, the level of the margarine group tended to

be lower in spite of its higher intake of polyunsaturated fatty acids. Accordingly, the plasma lipid peroxide levels seemed to be influenced by the dietary VE intake and not by the levels of dietary polyunsaturated fatty acids in a short-term study as in the present case. Yet, as already reported [20], the ordinary diet supplemented with canned mackerel containing highly unsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids increased the plasma lipid peroxide level and decreased the plasma α-tocopherol level even in a week in human subjects. Therefore, linoleic acid, the main constituent of margarine, is not so efficient as highly unsaturated fatty acids in fish oils in such actions to increase plasma lipid peroxide level and to decrease the plasma VE level.

Since the VE nutritional status of the subjects who participated in our present study was marginally deficient at the beginning, even though the whole experimental period of 8 days was very short, it was possible to elucidate which blood components best reflected the changes in VE intakes in the process of recovery from marginal VE deficiency. However, this is a rather rare case among the studies in humans even if it is very valuable case. Hence, further study using human subjects with normal VE status and with a longer experimental period will be necessary. Furthermore, in the process leading to VE deficiency leukocytes have been shown to be a likely blood component to monitor rat VE status [35]. And also, α-tocopherol in buccal mucosal cells has been examined to assess human VE status [36]. Future investigations will also be focussed on these indices.

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