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Vitamin E in young and old human red blood cells *

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Young and old human red blood cells contain about the same amount of α-tocopherol, a compound which has previously been shown to be the major lipid-soluble, chain-breaking antioxidant present in such cells. Since red blood cells lose up to ca. 20% of lipid material from their membrane as they age, the α-tocopherol/membrane-lipid ratio actually rises with age rather than declining as might have been expected on the basis of the free radical theory of aging. The α-tocopherol/arachidonic acid moiety ratios increase in the order: young red blood cells < old red blood cells < plasma, which argues against the suggested membrane stabilizing effect of α-tocopherol/arachidonic acid moiety complexes.

Introduction

The red blood cell provides an excellent model for studying the process of cellular aging because young and old cells have different densities and so can be separated by careful centrifugation [1–15]. It has been found [10] that older red blood cells have increased hemoglobin and Na^+ concentrations relative to younger cells but decreased K^+, acetylcholine esterase, sialic acid, membrane protein, 2,3-diphosphoglycerate, glutamic-oxaloacetic transaminase [14], ATP, creatine [4,12], and phospholipid [2,3] concentrations, together with a decreased concentration of fatty acids of C_{20} or longer chain length relative to those C_{18} and shorter [3,6]. In addition, the activities of catalase, glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione peroxidase [15] and superoxide dismutase [13] have been shown to decline in older cells. During the aging process red blood cells decrease in size [5], losing about one-fifth of their membrane (probably as cytoplasm-filled micro-vesicles [16,17]) but little or no hemoglobin; they also become osmotically more fragile [1,8,18].

Despite the wealth of information about the cellular transformations that occur with aging, the actual mechanism of the aging process is still poorly understood. If the free radical theory of aging [19] has general validity it should, as has sometimes been assumed [20], be specifically applicable to red blood cells. We have previously shown that vitamin E (mainly α-tocopherol) is essentially the only lipid-soluble, chain-breaking antioxidant present in human plasma and red blood cell ghost membranes [21,22]. One might therefore expect that the older red blood cells would be depleted in vitamin E relative to the younger cells. Surprisingly, no data have been

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Data supplementary to this article are deposited with, and can be obtained from Elsevier Science Publishers BV/1000 BH Amsterdam, The Netherlands. Reference should be made to no.: BBA/DD/342/73220/860 (1986) 84–90. The supplementary information includes: Three tables containing the *O*-acyl fatty acid composition of human red blood cells separated by density differences, the plasma lipid concentrations and fatty acid composition from donors of different age, and a comparison of heptane-isopropanol and SDS lipid extractions of young and old red blood cells.

reported with regard to the relative concentrations of vitamin E in young and old red blood cells. The present study was undertaken to rectify this omission.

Materials and Methods

Fresh whole blood was obtained by venipuncture from seven male adult volunteers, aged 26–56 years, after an overnight fast. Two of the volunteers are currently taking either all-*rac*-α-tocopherol acetate (KI; 400 I.U. once per week) or (*RRR*)-α-tocopherol acetate (GB; 100 I.U. once per week) as a dietary supplement. The blood (100 ml.) was drawn into evacuated, sterile tubes coated with the anticoagulant, disodium ethylenediaminetetraacetate. Within half an hour of being drawn, the blood was centrifuged slowly (1500 rpm) for 10 min to remove the bulk of the plasma, platelets, and white blood cells. (All centrifugations were done at 4°C (except where noted otherwise) in a Sorvall RC2-B centrifuge equipped with an SS-34 rotor, 4.25 in. radius.) Plasma, obtained from the crude plasma fraction by centrifuging at 6000 rpm for 10 min, was stored at 4°C for 1 day prior to extraction of the lipid material. The crude red blood cell fraction was washed three times by the addition of 5 mM phosphate-buffered saline (pH 7.4) [23,24] followed by slow centrifugation (1500 rpm) for 10 min. After the final wash the red blood cells were packed by spinning at 10000 rpm for 10 min and the supernatant was removed. The packed cells were then washed with 5 mM phosphate-buffered saline containing 5 mM ascorbate (pH 7.0) and centrifuged at 10000 rpm for 10 min. After the supernatant had been removed, 20 ml of the packed cells were remixed with 10 vol.% of the same buffer. This red blood cell suspension (hematocrit ca. 90%) was then fractionated by density according to the procedure of Murphy [9]. (Note, the α-tocopherol does not survive this step if the ascorbate is not present.) The top 10% and bottom 10% (ca. 2 ml each) of the red blood cells were harvested after removal of the supernatant. Each fraction was diluted to a known volume (5 ml; hematocrit ca. 40–45%) with the ascorbate containing phosphate-buffered saline.

Samples of each red blood cell suspension were withdrawn and microhematocrits, red cell counts (measured with a hemocytometer), mean cell volume, and mean cell hemoglobin concentrations were all determined by standard methods [25].

The level of creatine was estimated according to the method of Griffiths [26] using 0.5 ml of each red blood cell suspension diluted to 1 ml with distilled water. Lipid was extracted from 0.5 ml of each red blood cell suspension by a solvent mixture of heptane-isopropanol (3 : 2, v/v) as described by Radin [27]. The heptane-isopropanol solvent was removed in vacuo and the lipid residue was taken up in 2.0 ml of *n*-heptane. Vitamin E extraction was performed on 3 ml of each red blood cell suspension by our new sodium dodecyl sulphate (SDS) method [28]. Lipids and vitamin E were extracted from 1 ml of plasma using 1 ml each of ethanol and *n*-heptane [22]. α-Tocopherol was measured as described earlier [28].

O-Acyl fatty acids and cholesterol were determined simultaneously by capillary gas chromatography after their conversion to the corresponding methyl esters and the *t*-butyldimethylsilyl ether, respectively. The fatty acid methyl esters were obtained in the usual way [28] by sodium methoxide-catalyzed transesterification of duplicate 0.8 ml samples of the lipid dissolved in *n*-heptane (corresponding to 0.1 ml or less packed red blood cell). Tripentadecanoin and triheptadecanoin (NuChek Prep, Elysian, MN.) were added as internal standards to the *n*-heptane extracts and the *n*-heptane was removed by evaporation under a stream of nitrogen prior to the addition of the sodium methoxide reagent. Silylation of cholesterol was carried out by evaporating down, under a stream of nitrogen, a hexane solution of fatty acid methyl esters and cholesterol obtained after the transesterification step, adding 150 μl of *t*-butyldimethylchlorosilane/imidazole reagent (Applied Science, State College, PA) to the residue and heating the mixture at 80°C for 20 min [29]. Excess reagent was removed by adding chloroform and washing three times with water. The chloroform layer was dried over sodium sulfate, decanted, and the chloroform removed under a stream of nitrogen. The residue was taken up in 0.5 ml of *n*-heptane. Samples (1 μl) were introduced into a 50 m × 0.25 mm inner diameter fused

silica capillary column containing a bonded 75% cyanopropyl methyl silicone phase (CPS-1; 0.25 μ film thickness; Quadrex Corp., New Haven, CT) by means of a Varian 1095/11095 on-column injector mounted on a Varian model 3700 gas chromatograph. The injector was programmed to rise from 180°C to 240°C at 60 Cdeg/min immediately after injection. The oven temperature was programmed to hold at 60°C for 1 min after injection, then rise to 235°C at 20 Cdeg/min and maintain that temperature for a further 10 min. The detector was set at 300°C and the helium flow rate was 3 ml/min.

Results

Despite the modification we made by harvesting the top 10% and bottom 10% (instead of 5% as suggested by Murphy [9]) of the fractionated packed cells, distinct biochemical differences exist between the two fractions which indicate that the cells in the top fraction are younger than those in the bottom one. Specifically, the creatine levels in the top fractions are considerably higher than in the bottom fraction [4,12] (see Table I). There are also differences in other red cell indices between the two fractions which are in good agreement with earlier reports [3,10] *. Lipid analysis showed that the top fractions contain more cholesterol and fatty acids than the bottom ones (Table I). (The top fractions were also usually found to contain more arachidonic acid, in agreement with published data [2,3,6]).

* It has been pointed out that normal values (see e.g., Ref. 25) for mean cell hemoglobin are 32–36 g/100 ml and for the number of cells/ml of packed cells $(1.08–1.22)\cdot 10^{10}$. Some of our values for these quantities (and for *O*-acyl fatty acid/cell and cholesterol/cell) may therefore be in error caused in part, at least, by difficulties in correctly counting cells with a hemocytometer. Fortunately, as also noted, all our more important conclusions are based on relative (lipid/lipid; young/old) data and should therefore be unaffected by these problems.

TABLE I

RED BLOOD CELL INDICES, *O*-ACYL FATTY ACID AND CHOLESTEROL OF HUMAN RED BLOOD CELLS SEPARATED BY DENSITY DIFFERENCES

Source	Age (y)	Creatine (μg/ml packed cells)		Mean cell hemoglobin (g/100 ml packed cells)		No. of cells/ml of packed cells ($\times 10^{-10}$)		*O*-Acyl fatty acid [b] (mol/cell) ($\times 10^{16}$)		Cholesterol [b] (mol/cell) ($\times 10^{16}$)	
		young [a]	old [a]	young	old	young	old	young	old	young	old
SC	26	10.46	6.51	40.7	49.4	1.13	1.36	6.01	5.33	3.29	2.99
		10.35	4.59	41.8	49.6	1.30	1.45	±0.21	±0.45	±0.12	±0.19
PY	32	6.89	4.41	34.2	41.3	1.25	1.30	7.89	7.25	3.95	3.33
		6.78	4.78	33.9	41.0	1.16	1.28	±0.48	±0.35	±0.12	±0.38
DG	37	8.30	4.36	33.8	40.6	1.31	1.34	4.98	4.74	2.91	2.89
		8.24	3.21	33.7	41.2	1.33	1.36	±0.07	±0.16	±0.21	±0.21
GB [c]	38	4.88	3.82	31.4	36.6	1.18	1.41	3.14 [d]	2.79 [d]	2.69 [d]	2.45 [d]
(100)		5.01	3.47	31.1	36.8	1.28	1.44	±0.20	±0.01	±0.06	±0.34
JW	43	9.94	2.14	36.8	46.2	1.08	1.25	5.90	5.73	3.23	2.50
		9.09	3.00	37.0	46.4	1.25	1.33	±0.28	±0.19	±0.08	±0.03
JV	52	8.61	2.38	30.1	34.7	1.28	1.39	4.71	4.14	2.38	2.21
		8.66	2.62	29.8	34.3	1.26	1.37	±0.17	±0.13	±0.10	±0.06
KI [c]	56	8.43	2.17	41.5	51.7	1.27	1.37	5.34	4.72	2.80	2.41
(400)		9.42	2.99	41.7	51.9	1.20	1.38	±0.21	±0.23	±0.14	±0.13

[a] Young = top 10%; old = bottom 10%.

[b] Composite quantity derived from the mean of two measurements for each variable. Propagated error is calculated from the deviation from the mean for each quantity.

[c] Numbers in parentheses represent the amount (I.U.) of (*RRR*)-α-tocopherol acetate (GB) or all-*rac*-α-tocopherol acetate (KI) taken once per week by the volunteer as a dietary supplement.

[d] Lipid obtained by SDS method. Not all of the lipid is extracted by this procedure (see text).

TABLE II

α-TOCOPHEROL CONCENTRATIONS IN HUMAN RED BLOOD CELLS SEPARATED BY DENSITY DIFFERENCES

α-Tocopherol (α-T) concentrations were measured by HPLC using fluorescence detection. See also footnote b, Table I. α-T/lipid is a mole ratio. Lipid is the molar sum of *O*-acyl fatty acids and cholesterol (see Table I).

Source	α-T (mol/cell)($\times 10^{19}$)		α-T/lipid ($\times 10^{4}$)	
	young	old	young	old
SC	2.24±0.12	2.96±0.11	2.4±0.1	3.5±0.2
PY	3.00±0.10	3.82±0.14	2.5±0.2	3.6±0.3
DG	2.90±0.03	3.35±0.03	3.7±0.1	4.4±0.1
GB [a] (100)	3.15±0.01	3.11±0.10	5.4±0.2 [b]	5.9±0.4 [b]
JW	2.39±0.08	2.38±0.15	2.6±0.1	2.9±0.2
JV	2.18±0.09	2.51±0.11	3.1±0.2	4.0±0.2
KI [a] (400)	1.98±0.03	2.08±0.04	2.4±0.1	2.9±0.1

[a] See footnote c in Table I.
[b] See footnote d in Table I.

The older cells appear to contain as much or even more α-tocopherol/cell than do the younger cells (Table II). This is more apparent if we consider the α-tocopherol/lipid ratios in the old and young cells (Table II). Cells from the middle fraction of one blood sample (JV) are of intermediate age to judge, for example, from their creatine level (Table III) and the α-tocopherol level in these cells is also intermediate between the values found for the young and old cells. Thus, all our results indicate that in red blood cells the α-tocopherol/lipid ratio increases with increasing cell age.

TABLE III

CELL AND LIPID PARAMETERS FOR HUMAN RED BLOOD CELLS OF INTERMEDIATE AGE

Blood was donated by JV. Intermediate age blood was obtained by harvesting the middle fraction of blood separated by density.

Parameter	
Creatine (μg/ml packed cells)	4.28, 4.45
Mean cell hemoglobin concentration (g/100 ml packed cells)	30.5, 31.3
No. of cells/ml packed cells ($\times 10^{-10}$)	1.32, 1.34
O-Acyl fatty acid (mol/cell)($\times 10^{16}$)	4.18±0.35 [a]
Cholesterol (mol/cell)($\times 10^{16}$)	2.40±0.18 [a]
α-Tocopherol (mol/cell)($\times 10^{19}$)	2.18±0.12 [a]
α-Tocopherol/lipid ($\times 10^{4}$)	3.3 ±0.3 [a]

[a] See footnote b in Table I.

The concentration of α-tocopherol in the red blood cell membrane is affected by the concentration in the plasma and by the concentration of plasma lipids [28,30]. For this reason we determined the plasma α-tocopherol concentrations, lipid concentrations and compositions of our donors. Partition coefficients for α-tocopherol between plasma and young red blood cells and be-

TABLE IV

PARITION COEFFICIENTS OF α-TOCOPHEROL BETWEEN PLASMA AND YOUNG AND OLD RED BLOOD CELLS.

See footnotes a and b in Table I. Results are calculated from mole ratios. α-T/lipid is a mole ratio. Lipid is the molar sum of *O*-acyl fatty acids and cholesterol (see Table I). (Molar quantities of cholesterol and *O*-acyl fatty acids are given in Table VII in the Supplementary Material.) RBC, red blood cells.

Source	$(\alpha\text{-T/lipid})_{plasma}$ ($\times 10^{4}$)	$\frac{(\alpha\text{-T/lipid})_{plasma}}{(\alpha\text{-T/lipid})_{young\ RBC}}$	$\frac{(\alpha\text{-T/lipid})_{plasma}}{(\alpha\text{-T/lipid})_{old\ RBC}}$
SC	15.5±0.4	6.5±0.3	4.4±0.3
PY	12.2±0.9	4.9±0.5	3.4±0.4
DG	19.8±0.5	5.4±0.2	4.5±0.2
GD(100) [a]	24.1±0.5	4.5±0.2	4.1±0.3
JW	20.2±1.7	7.8±0.7	7.0±0.8
JV	22.4±1.7	7.2±0.7	5.6±0.5
KI(400) [a]	15.0±1.1	6.3±0.5	5.2±0.4
Mean±S.E.	18.5±1.6	6.1±0.5	4.9±0.5

[a] See footnote c in Table I.

TABLE V

MOLAR RATIOS OF ARACHIDONIC ACID IN RED BLOOD CELLS (RBC) AND PLASMA AND OF α-TOCOPHEROL TO ARACHIDONIC ACID IN HUMAN PLASMA AND YOUNG AND OLD RBC [a]

Source	20:4 (RBC)/20:4 (plasma)		α-Tocopherol/arachidonic acid ($\times 10^3$)		
	young [b] RBC	old [b] RBC	plasma	young RBC [b]	old RBC [b]
SC	2.8±0.1	2.6±0.1	38±2	2.0±0.2	3.2±0.3
PY	2.2±0.1	2.3±0.1	23±1	2.1±0.2	2.7±0.2
DG	2.6±0.2	2.3±0.2	48±3	3.5±0.1	4.9±0.3
GB [c] (100)	2.1±0.1	2.2±0.1	47±2	5.7±0.4 [d]	6.0±0.2 [d]
JW	4.8±0.2	4.0±0.2	88±8	2.5±0.2	3.1±0.2
JV	2.8±0.1	2.5±0.1	50±4	2.3±0.2	3.5±0.2
KI [c] (400)	2.5±0.1	2.2±0.1	30±3	1.9±0.1	2.6±0.2

[a] See footnote b in Table I.
[b] See footnote a in Table I.
[c] See footnote c in Table I.
[d] See footnote d in Table I.

tween plasma and old red blood cells are given in Table IV.

In Table V the molar ratios of α-tocopherol to arachidonic acid in plasma and red blood cells are given. These data are relevant to the suggestion that α-tocopherol stabilizes membranes by complex formation with arachidonic acid moieties [31–34], vide infra.

Although we reported earlier that the SDS method yielded recoveries of lipid from red blood cells that were equal to or better than those obtained by using either the Folch or Bligh-Dyer methods [28] we have since discovered that the heptane-isopropanol method [27] gives 1.5–2.4 times better yields of *O*-acyl lipid. However, it should be noted that the recovery of cholesterol by this method was no better than the recovery by the SDS method. Moreover, the recovery of α-tocopherol by the heptane-isopropanol method was extremely poor; so poor, in fact, that this method should not be employed for vitamin E analyses.

Discussion

The aging of red blood cells does not lead to any net depletion of their α-tocopherol content. On the contrary, the membrane lipids in the older cells are significantly enriched in vitamin E.

As was mentioned in the Introduction, red blood cells lose about 20% of their membrane lipid during their life span. In vitro studies have shown that micro-vesicles are released by the aging red blood cells [16] and that this process can be induced by endogenous ATP depletion [17], or by incubation with liposomes of phosphatidylcholines containing polyunsaturated fatty acyl chains [35]. In the latter case, microvesicle release could be inhibited by free radical scavengers and by the incorporation of α-tocopherol into the liposomes [35], which certainly suggests that lipid peroxidation is involved in the membrane vesiculation process. It therefore appears reasonable to assume that lipid peroxidation precedes vesiculation and actually occurs during the in vivo aging of red blood cells. If this assumption is correct then the α-tocopherol that is oxidized by the lipid peroxyl radicals may be partially or completely 'regenerated' by water-soluble reducing agents that are present in the plasma (e.g., ascorbate), as has been shown to occur in model systems [36–40]. Any oxidized α-tocopherol that escapes regeneration must be replaced from the plasma lipoprotein pool of α-tocopherol.

It has been suggested [31–34] that α-tocopherol exerts its membrane-stabilizing effect via complex formation with arachidonyl moieties of membrane phospholipids, with the methyl 'branches' on the

phytyl side chain (or on the chroman ring [41]) fitting into the *cis*-double bond 'pockets' of the polyunsaturated fatty acids. One might therefore expect that lipids rich in arachidonic acid residues would also be rich in α-tocopherol. However, this expectation is certainly not realized for the lipids in blood (Table V). Thus, the red blood cell membrane lipids contain much less α-tocopherol than do the plasma lipids [22] (see also Tables II and IV), but they contain much more arachidonate (Table V). While it might be suggested that the lipoprotein and membrane environments are too different for this result to argue against complex formation, the same could not be said so readily about red blood cell membranes. Nevertheless, the young cells have a lower ratio of α-tocopherol to arachidonate than the old cells (Table V). We would therefore argue that α-tocopherol does not stabilize membranes by forming complexes – whether static [31,32,34] or dynamic [33] – with polyunsaturated fatty acids. (No relationship implying stabilization is apparent with 18 : 2 or 22 : 6 either.) Our arguments are supported by the fact that the old cells contain a higher concentration of α-tocopherol (relative to lipid) than the young cells but, nevertheless, are osmotically more fragile than the young cells [8,19].

Our results also tend to argue against the existence of specific binding sites for α-tocopherol within the lipid portion of red blood cell membranes. If such sites existed one might have expected to find a higher concentration of α-tocopherol in red blood cell membrane lipids than in the plasma lipids, rather than the reverse. On the other hand, the existence of such sites, particularly if they are at least partly protein in nature [42], could explain why α-tocopherol is not lost as the cell ages and sheds membrane microvesicles. It remains to be seen if these microvesicles contain vitamin E.

None of our results show any trend with the age of the donor (26–56 years). It is known that the red blood cells of aging rodents have a shorter mean half-life than those of younger animals together with reduced levels of free thiol, glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase, and glucose-6-phosphate dehydrogenase [13,15,43]. The red blood cell membranes of the older animals would therefore appear to be more susceptible to peroxidative damage than those of the younger animals. It has been found that in humans α-tocopherol concentrations decrease with age of the subject in platelets but not in plasma [44], which means that the $[(\alpha\text{-tocopherol})_{plasma}/(\alpha\text{-tocopherol})_{platelet}]$ ratio increases with age. Based on our own, very limited, study the $[(\alpha\text{-tocopherol})_{plasma}/(\alpha\text{-tocopherol})_{red\ blood\ cell}]$ ratio is not age dependent.

Finally, we note that the paradox that vitamin E increases with age is not without a parallel. Specifically, Kunert and Edever [45] have very recently reported that the content of vitamin E in beech leaves and in fir needles increases with age.

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