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In vitro immune response of human peripheral blood cells to vitamins C and E

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Abstract

Since oxygen free radicals exert a noxious effect on cell functions, the purpose of the study was to examine the influence of the antioxidant vitamins C and E on the phagocytic capacity, apoptotic death, production of TNFα and IL-10 by human peripheral blood cells. In addition, an attempt to find a correlation between the effect of these vitamins on apoptosis and DNA synthesis was carried out. Peripheral white blood cells obtained from 27 healthy volunteers were incubated for 24 hr without and with vitamins C and E at doses extrapolated from clinical practice. Incubation of cells with vit. C caused a significant increase in the number of latex particles internalized by each individual polymorphonuclear cell, but not by monocytes. Both vitamins did not change the number of cells capable for phagocytosis. By the method of propidium iodide staining for detection of apoptosis, incubation of the cells with 0.2 mg/mL vit. C for 24 hrs caused a 39% increase in the percentage of apoptotic cells, as compared to those kept at the same incubation conditions without vitamin. 0.125 mg/mL of vit. E did not affect the percentage of apoptotic cells. On the other hand, applying the caspase-3 method for apoptosis detection, vitamins C and E did not affect the caspase-3 activity. Both vitamins caused an inhibition of ^{3}H-TdR incorporation, which was dose-dependent for vit. C. Concentrations of the vitamins lower than those mentioned above did not alter DNA synthesis. While TNFα production was not affected by both vitamins, the spontaneous secretion of IL-10 was dose-dependently reduced by vit. C but remained unaltered following incubation with vit. E. The results, although observed in vitro, might be of importance when those vitamins are administered to healthy subjects.

Keywords: Vitamins; Phagocytosis; Apoptosis

1. Introduction

An increasing number of reports have drawn attention to the reactive oxygen species as inducers of potential cell damage. It has been shown that a row of various free radicals can alter biological molecules including DNA strands [1,2]. Therefore, antioxidants exerting a protective effect on human cells, particularly those related to the proper function of the immune system may play an important role in the immune defense. In this sense, vitamins known as antioxidants, such as C and E, have gained significant reputation as health promoters and protectors of the immune cell functions [3]. Studies in recent years have shown that these vitamins improve the phagocytic function of polymorphonuclear cells in humans, especially at advanced age, as well as in cattle and fish [4-8]. Chai et al. [9] have observed a protective effect of vitamin E on the phagocytic capacity of human polymorphonuclear cells in patients with severe burns. Given the important role of vitamin C and E as preventers of oxidative stress in the normal phagocytic function, one would expect that they might affect the regulation of apoptosis with its consequences on cell homeostasis.

Therefore, the objective of this report was to examine the in vitro effect of the vitamins C and E at doses extrapolated from clinical practice on the phagocytic capacity and apoptosis of peripheral blood polymorphonuclear cells. An attempt was made to find a correlation between the effect of the vitamins on apoptosis and DNA synthesis. In addition, considering the role of these two vitamins in immune defense, their effect of the production of the pro-inflammatory cytokine TNFα and anti-inflammatory interleukin 10 (IL-

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10) by peripheral blood mononuclear cells (PBMC) was tested.

2. Materials and methods

The Helsinki committee of the Rabin Medical Center approved the project.

2.1. Cells

Mononuclear and polymorphonuclear cells were isolated from peripheral venous blood of 27 healthy volunteers using a histopaque (Sigma) gradient centrifugation. Mononuclear cells were suspended in RPMI-1640 medium containing 1% penicillin, streptomycin and nystatin, and supplemented with 10% fetal calf serum (complete medium, CM) (Biological Industries, Beit Haemek, Israel).

2.2. Phagocytosis of latex particles

0.8 mL of cell suspension (2×10^6 cells/mL) in condition medium (CM) was incubated without or with 0.2 mg/mL vit C, or 0.125 mg/mL vit E for 60 min. The dosage of the vitamins was extrapolated from that used in clinical practice. The engulfing capacity of the phagocytes was examined by addition of 0.2 mL of a 5% suspension of uniform polystyrene latex particles (0.8 μm in diameter, Difco, Detroit, MI). The cells were incubated with latex particles for 60 min. at 37°C, then sedimented, smeared on glass slides and stained using May-Grunwald-Giemsa dye. The number of cells that internalized latex particles, as well as the number of latex particles phagocytized by each individual cell was counted using a light microscope. At least 100 cells from each experimental point were examined.

2.3. Effect of vit. C and E on apoptosis

2.3.1. Propidium iodide staining

3×10^6 PBMC suspended in 1 mL FCS-M were incubated for 24 hrs. without and with 0.2 mg/mL vit C, or 0.125 mg/mL vit E. At the end of the incubation period, the cells were collected, washed in phosphate buffered saline (PBS), and fixed in 70% ethanol for 30 min at 4°C. Detection of apoptotic cells was carried out by a flow cytometric assay using propidium iodide staining [10]. In short, the cell pellet was suspended in a 50 μg/mL propidium iodide (Sigma) solution in PBS containing 100 μg/mL RNAse (Sigma) for one hour. The propidium iodide fluorescence of individual nuclei in 10,000 cells was examined with a flow cytometer FACScan (Becton-Dickinson, Singapore) at 580 nm, allowing detection of apoptosis as hypodiploid DNA containing cells.

2.3.2. Detection of caspase-3 level

4×10^6/mL PBMC were incubated in the absence or presence of 0.2 mg/mL vit. C or 0.125 mg/mL of vit. E. At the end of the incubation period, the cells were collected, sedimented by centrifugation and the level of caspase-3 was determined using Caspase-3/CPP32 calorimetric assay kit (BioVision, Palo Alto, CA). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The pNA light emission was quantified using a microtiter plate reader at 405 nm. The absorbency of pNA from samples treated with vitamins was compared with untreated controls. The cells were lysed using Cel Lysis Buffer and the protein concentration in the supernatant was assayed using Bradford reagent. Caspase-3 level in each sample was determined in 200 μg protein.

2.4. Effect of vit C and E on IL-10 and TNFα production

2×10^6 PBMC from all subjects, suspended in 1 mL FCS-M were incubated with vit. C at concentrations of 0.002, 0.02 and 0.2 mg/mL and vit. E at concentrations of 0.00125, 0.0125 and 0.125 mg/mL added at the onset of the cultures. Cultures incubated in the absence of the vitamins served as controls. The culture media were collected at 24 hr, the cells were removed by centrifugation and the supernatants were kept at −70°C until assayed.

The concentration of the cytokines in the supernatants was tested using ELISA kits specific for human IL-10 and TNFα (Pharmingen, San Diego, CA) as detailed in the guide-line provided by the manufacturer. The detection level in the assay was 15 pg/mL for IL-10 and 30 pg/mL for TNFα.

2.5. Effect of vit. C and E on ^{3}H-thymidine incorporation

1 mL aliquots of PBMC from 12 individuals (2×10^6/ml) were incubated for 24 without or with vit. C or vit. E in duplicates at the concentrations indicated above. At the end of the incubation period, 10 L of ^{3}H-thymidine (10 Ci/mL, 49 μCi/mmole) was added to each tube for additional 3 hr. The cells were then washed twice with cold saline, and trichloroacetic acid-precipitable counts were obtained using an LKB liquid scintillation counter (model 3380).

2.6. Statistical evaluation

Statistical analysis was performed using one way analysis of variance (ANOVA) and the paired or independent *t*-test. The results are expressed as a mean ± SEM.

3. Results

3.1. Effect of vit. C and E on phagocytosis

(Fig. 1). The percentage of polymorphonuclear cells capable to internalize latex particles was 73.4 ± 4.1, 78.2 ± 3.6 and 88.5 ± 3.0 for cells incubated with CM, vit. C and

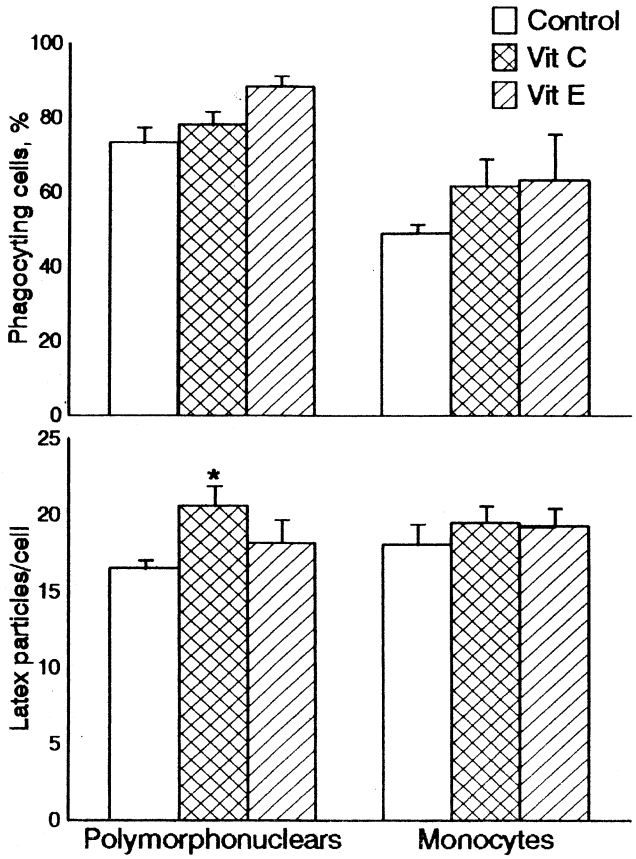

Fig. 1. The in vitro effect of vitamins C and E on the phagocytic capacity of human peripheral blood white cells (*$P < 0.001$). 2×10^6 cells were incubated without or with 0.2 mg/mL vit C, or 0.125 mg/mL vit. E for 60 min. and thereafter with 0.2 mL. of a 5% latex particles for additional 60 min. The number of cells engulfing latex particles and the number of particles internalized by each individual cell was counted with a light microscope.

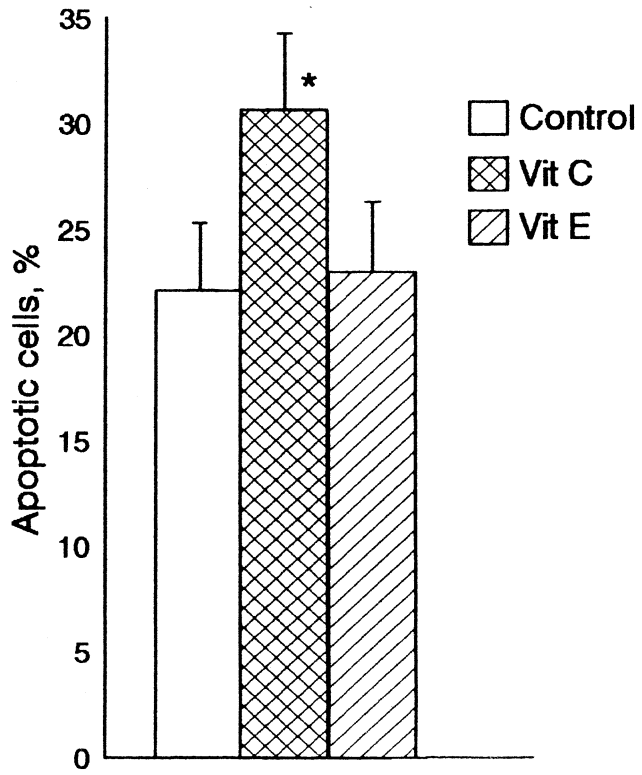

Fig. 2. Effect of vitamin C and E on the apoptotic response of human peripheral blood white cells ($n = 27$). 3×10^6 cells were incubated without or with 0.2 mg/mL vit C, or 0.125 mg/mL vit. E for 24 hr. At the end of the incubation period the cells were stained with propidium iodide and the fluorescence was detected with a flow cytometer FACScan. While vit. C caused a significant increase in the percentage of apoptotic cells, vit. E did not exert any effect on that cell function. *$P < 0.02$.

vit. E respectively. The percentage of phagocyting monocytes incubated at the same conditions was 49.0 ± 2.0, 61.8 ± 7.0 and 63.3 ± 11.9 respectively. On the other hand the number of particles engulfed by each polymorphonuclear cell was increased from 16.5 to 20.6 particles/cell after incubation with vit. C ($P < 0.001$), but it did not change following incubation with vit. E. Both vitamins did not affect the number of latex particles phagocytized by each individual monocyte.

3.2. Effect of vit. C and E on apoptosis

3.2.1. Propidium iodide staining

(Fig. 2). Incubation of PBMC with 0.2 mg/mL vit. C caused 39% increase in the percentage of apoptotic cells as compared with controls ($P < 0.02$). The number of apoptotic cells was not significantly different following incubation with 0.125 mg/mL vit. E.

3.2.2. Caspase-3 level.

The level of caspase-3 in mononuclear cells incubated for 24 hrs without or with 0.2 mg/mL vit. C or 0.125 mg/mL vit. E was similar (0.205 ± 0.022 nm, 0.202 ± 0.022 nm and 0.207 ± 0.023 nm/200 μg protein, respectively).

3.3. Effect of vit. C and E on ^{3}H-thymidine incorporation

Incubation of PBMC with vit. C showed a dose dependent inhibition of ^{3}H-TdR incorporation ($P < 0.01$). Concentrations of 0.02 and 0.2 mg/mL vit. C caused 22% and 50% inhibition of ^{3}H-TdR incorporation respectively ($P < 0.05$; $P < 0.001$, Fig. 3). Cells incubated with vit E, showed a reduced incorporation of thymidine ($P < 0.05$). At a concentration of 0.125 mg/mL thymidine incorporation was decreased by 22% ($P < 0.002$). Lower concentrations of both vitamins did not affect DNA synthesis.

3.4. Effect of vit. C and E on IL-10 and TNFα production

Following incubation of PBMC with 0.02 and 0.2 mg/mL of vit. C the spontaneous secretion of IL-10 was reduced by 25% and 31% respectively ($P < 0.05$, Fig. 4).

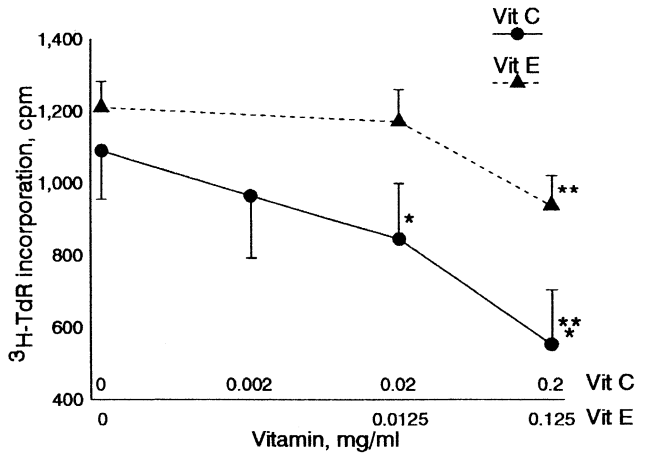

Fig. 3. Effect of vitamin C and E on ^{3}H-thymidine incorporation into human peripheral blood white cells ($n = 12$). 2×10^6 cells were incubated without or with 0.2 mg/mL vit C, or 0.125 mg/mL vit. E for 24 hr and thereafter with 10 μCi of ^{3}H-thymidine. In both cases a dose response was observed. $^*P < 0.05$, $^{**}P < 0.002$. $^{***}P < 0.001$.

The production of IL-10 was not affected either by lower doses of vit. C, or by the three concentrations of vit. E tested in this study. Both vitamins did not exert any effect on TNFα production.

4. Discussion

The results of the study indicate that incubation of human polymorphonuclear cells with vit. C caused a significant increase in number of latex particles phagocytized by each individual cell, whereas the number of cells capable for phagocytosis remained unchanged. The phagocytic function of the monocytes remained unaffected. As for vit. E, we could not show any effect of this antioxidant on the phagocytic capacity of both polymorphonuclear cells and monocytes. The reports in the literature concerning the effect of these two vitamins on the engulfing capacity of the phagocytes are rather controversial. On one hand, researchers have reported on an enhancing effect of the vitamins on phagocytic activity [6-9]. On the other hand, Andreasen et al. [11] could not observe a stimulatory effect of vit. C on the phagocytic activity of chicken heterophils in vitro, although treatment with ascorbic acid did induce a significant increase in bacterial killing. Hogan et al. [12] have shown that neutrophils from cows treated with vit. E exerted a greater intracellular kill of bacteria in comparison with controls, but their phagocytic index and percentage of phagocyting cells remained unchanged.

A similar ambiguity exists about the effect of vitamins C and E on programmed cell death. Yu et al. [13] induced apoptosis in murine EL4 T lymphoma cells by treatment with vit. E succinate. Satoh et al. [14] have shown that vitamins C and E exert a cooperative action in inducing apoptosis in human promyelocytic leukemic HL-60 cells,

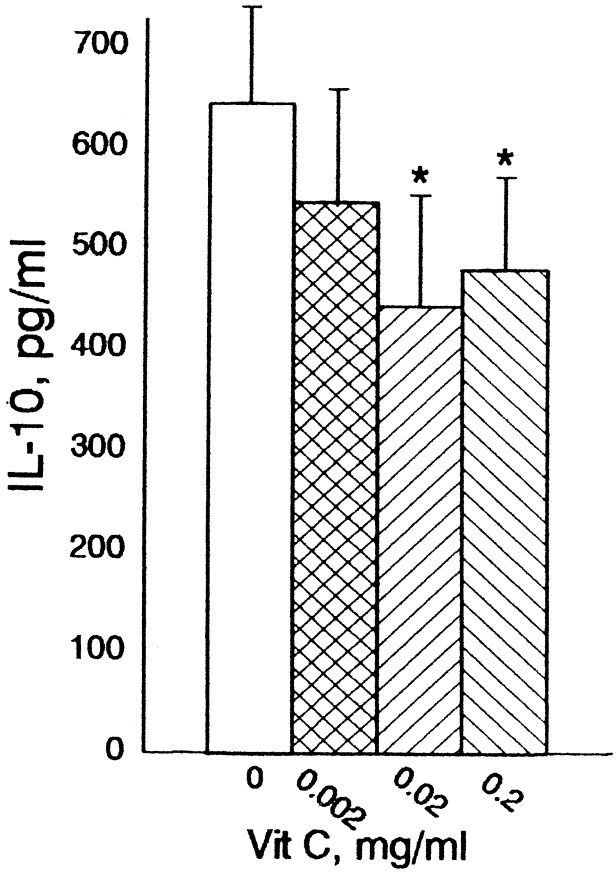

Fig. 4. A dose response effect of vit. C on IL-10 production by human peripheral blood mononuclear cells ($n = 27$). 2×10^6 cells were incubated without or with vit C, or vit. E for 24 hr at the indicated concentrations. The concentration of IL10 was detected as detailed in *Materials and Methods*. $^*P < 0.05$.

whereas Witeberg et al. [15] reported that loading these cells with vit. C provides a marked protection from apoptosis. An inhibitory effect of antioxidant vitamins on apoptosis has been observed after exposure of HL-60 myeloid leukemia cells and mouse splenocytes to ionizing irradiation [16,17]. Since oxidative stress may induce abnormal apoptosis, it is conceivable that antioxidants modified programmed cell death by a repair of its effect [18].

In the present study, when apoptosis was examined by the propidium iodide test, vit. C caused a marked increase in the number of apoptotic PBMC, indicating DNA fragmentation, whereas incubation with vit. E did not show any effect. On the other hand, when apoptosis was detected by the caspase-3 method, the enzyme activity in the cells following incubation with both vitamins did not change. This observation suggests the existence of a caspase-3 independent pathway for regulation of programmed cell death following incubation with vit. C. A similar mechanism for explanation of the apoptotic effect of nitric oxide in thymocytes via a caspase-1 dependent, but a caspase-3 independent pathway has been described by Zhou et al. [19]. The inductive effect of the vitamins on apoptosis of normal

PBMC suggests a suppressive effect of these two antioxidants on DNA synthesis. Indeed, both vitamins caused a dose dependent inhibition of ^{3}H-thymidine incorporation in PBMC, an effect more expressed when the cells were incubated with vit. C. However, in the case with vit. E we could not find a correlation between increased apoptosis and decreased ^{3}H-thymidine incorporation. The inhibitory effect of vit. C on thymidine incorporation into DNA with a subsequent decrease in cell viability has been demonstrated in prostate carcinoma cells [20]. It was implied that the inhibition of cell division induced by a S/G2 block in the cell cycle proceeds through generation of unidentified free radicals [21,22]. A similar inhibition of DNA synthesis has been observed in erythroleukemia, prostate and human breast cancer cells following treatment with vit. E [23,24]. Lee et al. [25] suggest that the DNA damage caused in vivo by vit. C may be due to its capacity to induce decomposition of lipid hydroperoxides which have been shown to generate genotoxins.

Previous studies have shown that both C and E vitamins affect cytokine production. Dietary supplementation with vit. E in mice infected with LP-BM5 retrovirus, restored the inhibited IL-2 and interferon gamma production by splenocytes and normalized the increased IL-6 and IL-10 production caused by retrovirus infection [26]. Healthy adults supplemented with vit. C and E showed increased production of IL-1β and TNF-α by their PBMC [27]. Schwager and Schulze [28] reported on alterations in IL-2 and IL-6 production by vit. C depleted pigs' PBMC.

The existence of a relationship between DNA synthesis and IL-10 has been established. Seppanen et al. [29] have observed an inhibition of DNA synthesis by IL-10 in ovarian and endometrial carcinoma cells. A similar effect of physiological doses of IL-10 on DNA synthesis by vascular smooth muscle cells was reported by Selzma et al. [30].

The spontaneous secretion of IL-10 following incubation of PBMC with vit. C in our work was dose-responsively suppressed, whereas that of TNFα remained unaffected. Vit. E at the doses used in the study did not affect IL-10 secretion. These results obtained in the present work are rather unexpected. Lack of effect on the number of cells capable for phagocytosis, the increase in number of apoptotic cells, the inhibited ^{3}H-thymidine incorporation, the lower IL-10 production caused by the antioxidants are not indicators that these vitamins are immune defenders.

On the other hand, these results were obtained in vitro and although the dosage of the vitamins was extrapolated from that applied in clinical practice the findings might be the outcome of dose dependence.

The distinct effects of vitamins C and E observed in the study may be explained by the fact that although both vitamins are potent reductants they may affect the ultimate oxidizing molecule by different pathways. For example, following reduction of lipid peroxide, vit. E becomes a radical that may be decomposed or reduced by a stronger reducing agent such as vit. C [31] a part of the mechanism designated as "network action of vitamins".

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