

A Short-Term Dietary Supplementation of High Doses of Vitamin E Increases T Helper 1 Cytokine Production in Patients with Advanced Colorectal Cancer¹

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ABSTRACT

Purpose: Patients with advanced cancer exhibit multifaceted defects in their immune capacity, which are likely to contribute to an increased susceptibility to infections and disease progression and to constitute a barrier to immunotherapeutic interventions. A chronic inflammatory condition associated with increased oxidative stress has been suggested as one of the responsible mechanisms behind the tumor-induced immune suppression. We, therefore, speculated that supplementation with the antioxidant vitamin E could enhance the immune functions in patients with advanced cancer.

Experimental Design: This hypothesis was here tested in twelve patients with colorectal cancer (Dukes' C and D) who, prior to intervention with chemo- or radiotherapy, received a daily dose of 750 mg of vitamin E during a period of 2 weeks.

Results: Short-term supplementation with high doses of dietary vitamin E leads to increased CD4:CD8 ratios and to enhanced capacity by their T cells to produce the T helper 1 cytokines interleukin 2 and IFN- γ . In 10 of 12 patients, an increase of 10% or more (average, 22%) in the number of T cells producing interleukin 2 was seen after 2 weeks of vitamin E supplementation, as compared with peripheral blood monocyte samples taken before treatment ($P = 0.02$).

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Interestingly, there seemed to be a more pronounced stimulatory effect by vitamin E on naïve (CD45RA⁺) T helper cells as compared with T cells with a memory/activated phenotype.

Conclusions: Dietary vitamin E may be used to improve the immune functions in patients with advanced cancer, as a supplement to more specific immune interventions.

INTRODUCTION

Patients and experimental animals with advanced cancer often exhibit a poorly functioning immune system (1, 2), manifested by anergy to skin-test antigens (3), decreased T-cell proliferation (4, 5), alterations in signal transducing molecules (6–11), reduced CD4:CD8 ratios (12, 13) and deficient production of T helper 1 cytokines (14–16). These alterations correlate with the severity of the disease and with poor survival (6, 17, 18). Removal of tumor burden by surgical resection was associated with normalization of cytokine production capacity in colorectal cancer patients (15). Furthermore, there is evidence for increased apoptosis among CD8⁺ T cells in PBLs³ from cancer patients (19–21) and from mice with experimental tumors (22). Tumor-induced immune suppression could contribute to the spreading of the disease and constitute a barrier to immunotherapeutic interventions.

Multiple factors may contribute to tumor-induced immune suppression. These include Fas-FasL interaction leading to T-cell apoptosis, shown to involve caspase 3-mediated cleavage of CD3 ζ (23), as well as tumor-derived gangliosides inducing defective nuclear factor- κ B activation in renal cell carcinoma lines (24). Recently several observations indicate that a chronic inflammatory condition develops in patients with advanced cancer, causing oxidative stress that can shut off immune functions, including those of T and NK cells. Free oxygen radicals produced by macrophages isolated from metastatic lymph nodes or from mice with experimental tumors were able to inhibit non-specific and tumor-specific cytotoxicity and down-regulate signal transduction molecules (25–27). Monocytes recovered from human PBMCs can inhibit autologous NK cell-mediated cytotoxicity via secretion of H₂O₂, leading to the induction of apoptosis (28). In addition, macrophage-derived nitric oxide reduces the phosphorylation and activation of JAK3/STAT5 signal transduction proteins, thus inhibiting the proliferative responses of T cells to IL-2 (29). Furthermore we recently reported that micromolar levels of hydrogen peroxide selec-

³ The abbreviations used are: PBL, peripheral blood lymphocyte; PMA, phorbol 12-myristate 13-acetate; PBMC, peripheral blood monocyte; RDA, recommended dietary allowance; mAb, monoclonal antibody; NK, natural killer; IL, interleukin; Th1, T helper 1.

tively inhibited Th1 cytokine production in memory/activated T cells and that this inhibition correlated with the blocking of nuclear factor- κ B activation (30).

Therapeutic interventions aimed to protect the immune system in cancer patients from damage caused by oxidative stress may, therefore, enhance their immune competence. This hypothesis was here tested in patients with advanced colorectal cancer, a patient group known to have defects in their capacity to produce Th1 cytokines in response to mitogen stimulation (14, 15, 31). Twelve patients with colorectal cancer [Dukes' C (2 patients) or D (10 patients)] were given high doses of dietary vitamin E in combination with vitamin C and selenium at RDA levels during a period of 2 weeks. Vitamin C and selenium were included for their capacity to recycle vitamin E, thus allowing vitamin E to function optimally (32–35). Changes in the capacity of patients' lymphocytes to respond to mitogen stimulation were evaluated. It was demonstrated that all subsets of T cells ($CD4^+$, $CD8^+$, $CD45RO^+$, and $CD45RO^-$) had enhanced capacity to produce IL-2 after 2 weeks of vitamin E treatment. Furthermore, significantly more cytotoxic T cells ($CD8^+$) were capable of IFN- γ production after treatment. Possible mechanisms behind the immunostimulatory effect of vitamin E and the role of the Th1 cytokines in tumor immunity are discussed. It is suggested that vitamin E may be used as an adjuvant to more specific immunotherapy that is dependent on a functional Th1 response.

PATIENTS AND METHODS

Eligibility and Exclusion Criteria. Patients with histologically confirmed colorectal carcinoma in Dukes' stage C or D and a Karnofsky score above 70 were eligible for inclusion in the study. Patients also had a WBC count $>3.0 \times 10^9$ /liter, hemoglobin >100 g/liter, and platelets $>100 \times 10^9$ /liter. Patients were excluded from the trial based on the following exclusion criteria: patients who had been on substitution therapy with vitamin E, vitamin C, or any other antioxidant less than 3 weeks before the start of the investigation; patients with coagulation imbalance and/or anticoagulant therapy; patients with evidence of serious active bacterial or viral infections, or with a current serious medical or psychiatric condition that would prevent obtaining informed consent or treatment; patients on continuous therapy with corticosteroids or nonsteroidal anti-inflammatory drugs; patients undergoing antitumoral treatment with cytotoxic drugs or radiotherapy; patients with organ allografts or autoimmune disease, or with concurrent hematological malignancies.

Treatment Plan. All of the patients were treated as outpatients. They received a total daily dose of 750 mg of vitamin E (RDA, 8–10 mg), 60 μ g selenium, and 90 mg vitamin C. This dose was distributed as follows: (100 mg vitamin E; ACO AB, Sweden), 3 tablets in the morning and 4 in the evening, OxiGard (50 mg vitamin E, 60 μ g selenium, and 90 mg vitamin C; ACO AB, Sweden) 1 tablet every morning. Treatment period was 15 days. Patients underwent physical examinations on day 1 and 15.

Cells. Patients' PBLs were isolated by Ficoll Paque (Pharmacia Biotech AB; Uppsala, Sweden) gradient centrifugation for 20 min at 20°C before and after vitamin E treatment and

were frozen in FCS 10% DMSO (Sigma, Stockholm, Sweden) for later analyses of their capacity to produce cytokines. Coded samples obtained before and after vitamin E treatment were analyzed in parallel.

Lymphocyte Activation for the Cytokine-staining Experiments. PBMCs were thawed and washed, and the lymphocyte concentration was adjusted to 1×10^6 cells/ml. The cellular viability in all of the experiments was higher than 97% as determined with 7-aminoactinomycin D (Sigma). The lymphocytes were stimulated for 4 h at 37°C with PMA and Ionomycin (Sigma) in the presence of 10 μ g/ml Brefeldin (Sigma; Ref. 36).

Measurements of Plasma α -Tocopherol. Plasma was collected after the gradient centrifugation as described above and was frozen at -20°C . Plasma was protected from daylight until measurements were performed. The α -tocopherol quantification was performed by a reverse-phase high-performance liquid chromatography method as described elsewhere (37).

FACS Analysis. A four-color method was used. The cells were first stained for surface antigens (30 min at 4°C) with anti-CD45RO-FITC (Dakopatts), anti-CD8-PerCP, and anti-CD3-APC (BDIS Biosciences, Stockholm, Sweden). Thereafter, the lymphocytes were permeabilized with FACS-lysing solution and with FACS permeabilizing solution (BDIS Biosciences), and were stained for 10 min at room temperature in the dark with PE-conjugated mAbs directed to IL-2, IFN- γ , IL-10, and TNF- α , (BDIS Biosciences). The staining protocol included isotype controls for both surface and cytoplasmic staining. The stimulation and permeabilization procedures were checked by cytoplasmic staining for CD69 (BDIS Biosciences) and vimentin (Serotec, Oslo, Norway). After staining, the cells were fixed with CellFix (BDIS Biosciences). Acquisition was performed in less than 2 h. The flow cytometric measurements were performed on a FACS Calibur (Becton Dickinson, Stockholm, Sweden). The instrument performance was checked daily with bead calibrators QC Windows, CaliBRITE, and Q1000, and monthly with both beads and cells as described previously (38). Data of at least 10,000 cells/sample were collected. Data analysis was done with Cell Quest software (BDIS Biosciences) according to a standardized pattern-protocol. The background fluorescence was determined with markers applied on the isotype control cytograms and was in all cases $<1\%$. Because all analyzed cells were T cells ($CD3^+$), the $CD8^-$ lymphocytes were considered $CD4^+$, and the $CD45RO^-$ lymphocytes were considered $CD45RA^+$.

IFN- γ ELISA. PBMCs were thawed and washed, and the lymphocyte concentration was adjusted to 1×10^6 cells/ml. Cells were seeded in triplicate cultures in 24-well plates (Costar, Corning Incorporated) and stimulated for 24 h by OKT-3 or control antibody at 100 ng/ml. Supernatants were harvested and the IFN- γ content was measured by an ELISA. Briefly, 96-well plates (Maxisorp; Nalge Nunc International, Taastrup, Denmark) were coated with 4 μ g/ml of capturing antibody, anti-human IFN- γ (mAb 1-D1K; Mabtech AB, Stockholm, Sweden) at 4°C for 18 h and then washed two times in wash buffer (PBS with 0.05% Tween 20) and blocked in blocking solution (PBS with 1% BSA and 0.05% Tween) for 1 h at 37°C. Sample and standard were added to wells in triplicate (100 μ l/well) and incubated for 2 h at 37°C. Plates were washed and secondary

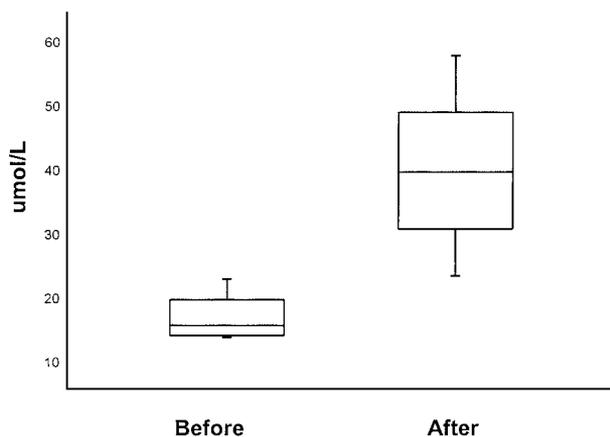


Fig. 1 Increased plasma α -tocopherol after vitamin E treatment. The plasma levels of α -tocopherol in $\mu\text{mol/liter}$, before and after vitamin E treatment are shown. Reference interval 13.5–36.8 $\mu\text{mol/liter}$. Boxes, 95% of the values; horizontal lines, medians; bars, SD; $P < 0.001$.

antibody (7-B6-1-Biotin; Mabtech AB) was added at 0.75 $\mu\text{g/ml}$ and incubated for 2 h at 37°C. Next, streptavidin-ALP (Mabtech AB, Stockholm) was added and plates were incubated for 30 min at 37°C. After washing, substrate (alkaline phosphatase pNPP; Sigma, Stockholm, Sweden) was added and incubated for 20 min before plates were read at 405 nm in an ELISA reader.

Statistical Analysis. Statistical analysis was performed using two-tailed, paired Student's *t* test. Significance was accepted at $P < 0.05$.

RESULTS

From the end of 1999 to September 2000, a total of 13 patients were included in this trial. The mean age was 66 years (range, 47–74). Eleven patients had tumors in stage Dukes' D and two in stage Dukes' C. Patient 12 developed ileus, after inclusion in the study, but before the start of treatment and was, therefore, not eligible. For the remaining twelve patients this regimen of vitamin E was well tolerated, and no side effects were reported. Patient 11 showed signs of infection at the time of the second bleeding but concluded the whole regimen and was, therefore, included in the analysis.

Increased Plasma α -Tocopherol Levels. As a result of the treatment, patients enhanced their plasma levels of α -tocopherol from 16.9 (range, 13.6–22.8) to 39.5 $\mu\text{mol/liter}$ (23.3–57.7), the reference interval for tocopherol being 13.5–36.8 $\mu\text{mol/liter}$ (Fig. 1). The increase in plasma α -tocopherol levels was statistically significant ($P < 0.001$). This analysis also served as a quantitative test for the treatment compliance among patients ensuring adequate intake of prescribed vitamins.

Increased IL-2 Production by CD4^+ and CD8^+ T Cells. The capacity of the patients' lymphocytes to produce cytokines after stimulation with PMA/Ionomycin was analyzed. In addition, cells were stained for various surface markers to enable the analyses of cytokine production in CD4/8 T cells of memory/activated phenotype (CD45RO^+) as compared with naïve cells (CD45RA^+ ; Fig. 2). In Fig. 3, typical density plots of the IL-2

production by T cells (CD3^+) before and after vitamin E treatment are shown. Data from a representative donor (patient 6) and from the donor who responded strongest (patient 9) are presented. In 10 of 12 patients, an increase of 10% or more in the number of T cells (CD3^+) producing IL-2 was seen after 2 weeks of vitamin E supplementation, as compared with PBL samples taken before treatment (Fig. 4A; $P = 0.02$). The average increase in the number of T cells capable of IL-2 production was 22%. The increase was almost identical in CD4^+ and CD8^+ T cells (Fig. 4, B and C). Only patient 11 had fewer cells capable of producing this cytokine after vitamin E treatment. Of note, this patient showed clinical signs of infection with an increased WBC count and elevated C-reactive protein (CRP) at the time of the second bleeding.

To further delineate the effects of vitamin E on the immune system, we analyzed the differences in IL-2 production between memory (CD45RO^+) and naïve (CD45RA^+) CD4 T cells. Although not statistically significant ($P = 0.13$), we found a strong tendency toward a more potent response to vitamin E by naïve CD4^+ T cells as compared with CD4^+ T cells with a memory/activated phenotype. Naïve CD4^+ T cells increased their capacity to produce IL-2 by an average of 32%, whereas memory/activated CD4^+ T cells increased their capacity by 19%. This tendency is in line with a recent study in old mice showing an enhanced IL-2 production by naïve T cells as a result of vitamin E (39). The increase in the number of cells producing IL-2 was, however, statistically significant for all of the subsets of T cells (CD4^+ , CD8^+ , CD45RO^+ , CD45RO^- ; $P < 0.05$).

There also seemed to be a diverse effect of vitamin E on Th1 versus Th2 responses, inasmuch as no statistically significant changes could be observed for IL-10 production. The average percentage of T cells that produced IL-10 in response to PMA/Ionomycin before vitamin E treatment was 16%; and after vitamin E treatment, it remained low (17%).

Increased Number of IFN- γ -producing CD8^+ CD45RO^+ T Cells after Vitamin E Treatment. The increases in IFN- γ responses after vitamin E administration were more variable and of less magnitude than for IL-2. Statistically significant increases in the number of IFN- γ -producing cells were seen in CD8^+ T cells with a memory/activated phenotype as defined by expression of CD45RO ($P < 0.05$; Fig. 5A). In 6 of 6 randomly selected donors, the increased number of IFN- γ -producing cells correlated with an enhanced IFN- γ release by PBMCs, stimulated by anti-CD3 as measured by ELISA ($P = 0.014$; Fig. 5B). Changes in TNF- α production were not statistically significant (data not shown).

Increased $\text{CD4}:\text{CD8}$ Ratios after Vitamin E Treatment. Decreased CD4 counts and, therefore, altered $\text{CD4}:\text{CD8}$ ratios were described to correlate with late-stage colorectal cancer (12) and advanced (stage III-IV) nodular-sclerosing Hodgkin disease (13) and may be an indicator of immune suppression. Here we report that overall $\text{CD4}:\text{CD8}$ ratios increased from 1.31 to 1.44 ($P < 0.05$) as a result of vitamin E treatment. Five of 12 patients had $\text{CD4}:\text{CD8}$ ratios below the reference interval (0.8–3.4; Ref. 38) before treatment. Further analysis of these five patients showed a more pronounced increase in $\text{CD4}:\text{CD8}$ ratios up to almost normalized levels (Fig. 6; $P < 0.05$). No significant

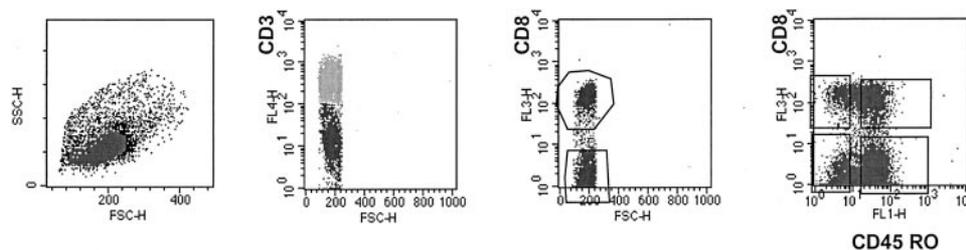


Fig. 2 Identification of the T-cell subsets. One representative staining of PBMC is shown. The density plots show how T-cell subsets were identified using a three-color surface staining. Gates were applied on all lymphocytes (light scatter cytogram), all CD3⁺ lymphocytes (FSC versus FL4), followed by subsequent gating on CD8⁺ and CD8⁻ subsets (FSC versus FL3). Each subset was further divided in two subpopulations: CD8⁺CD45RO⁺ and CD8⁺RO⁻, or CD8⁻CD45RO⁺ and CD8⁻CD45RO⁻ (FL1 versus FL3).

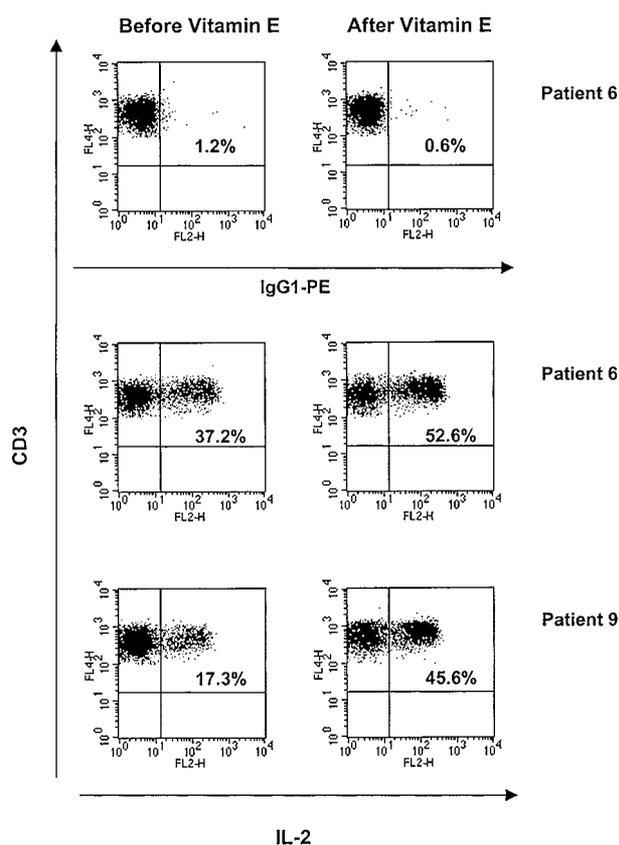


Fig. 3 Typical staining of IL-2 production in PMA/Ionomycin-activated T cells before and after vitamin E treatment. Either control antibody [IgG1-PE antibody (top)] or PE-labeled anti-IL-2 antibody (middle and bottom) was added as the fourth color (FL2). The number of T cells (CD3⁺) capable of producing IL-2 in patient 6 and 9 before (left column) and after (right column) vitamin E supplementation are shown.

changes in the number of NK cells or in the various subsets of T cells were observed (data not shown).

DISCUSSION

We report that a short-term dietary supplementation of high doses of vitamin E, in combination with vitamin C and selenium at RDA levels, given to patients with advanced colorectal cancer

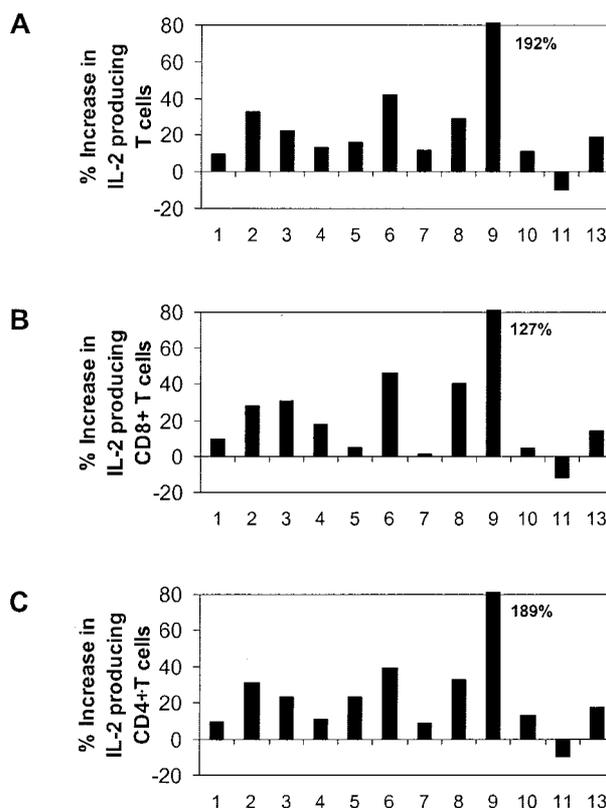


Fig. 4 Increased frequency of IL-2-producing CD4⁺ and CD8⁺ T cells in response to PMA/Ionomycin after a 2-week period of dietary vitamin E supplementation. The increase in the number of cells, in percentage, capable of producing various cytokines was based on intracellular staining and was calculated as follows:

$$\% \text{ increase} = 100$$

$$\times \frac{(\text{No. of cytokine-producing cells}/10,000 \text{ events after vitamin E}) - (\text{No. of cytokine-producing cells}/10,000 \text{ events before vitamin E})}{\text{No. of cytokine-producing cells}/10,000 \text{ events before vitamin E}}$$

The increase of IL-2-producing T cells (A), CD8⁺ T cells (B), and CD4⁺ T cells (C) are shown.

results in increased CD4:CD8 ratios and enhanced capacity of their T cells to produce IL-2 and IFN-γ in response to stimulation.

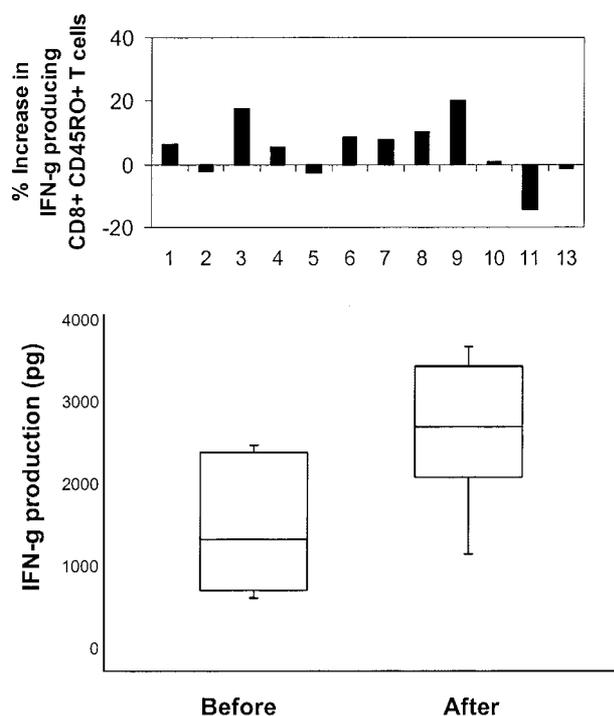


Fig. 5 A, significantly higher number of CD8⁺, CD45RO⁺ T cells capable of producing IFN- γ after vitamin E treatment. The increase, calculated as in Fig. 4, in the number of IFN- γ -producing CD8⁺, CD45RO⁺ T cells are shown. In B, the amount of IFN- γ (pg/100 μ l) produced by PBMCs stimulated by anti-CD3 mAb (OKT-3, 100 ng/ml) is shown before and after vitamin E supplementation. Boxes, 95% of the values; horizontal lines, medians; bars, SD; $P = 0.014$.

Vitamin E is a lipid-soluble antioxidant that stimulates T-helper cell activity in a dose-dependent manner in mice immunized with a hapten-carrier conjugate (40). Also, increased proliferative responses and IL-2 production has been described in old rats (41). Furthermore, vitamin E counteracts the age-related decline of the cellular immune response, both in humans and in mice, by lowering macrophage prostaglandin E₂ production (42–45). Vitamin E may decrease the incidence of prostate cancer in smoking men, as shown in a large primary prevention trial that is being followed up in the recently launched SELECT trial (the study of Selenium and Vitamin E Cancer Prevention Trial; Refs. 46, 47). Whether this effect is mediated via influence on the immune system or by other mechanisms is unknown.

In our study, vitamin E promoted both IL-2 and IFN- γ production by T cells. This was shown by staining for intracellular cytokines after stimulation with PMA/Ionomycin (Figs. 3, 4, and 5) and was confirmed in six randomly selected patients by ELISA experiments on supernatants from cultures stimulated with PMA/Ionomycin (data not shown) or OKT-3 (anti-CD3 mAb; Fig. 5B). In contrast, no significant induction of IL-10 production was observed as a result of vitamin E supplementation, favoring a Th1 response in these patients. We also describe a tendency toward a more pronounced effect of vitamin E in naïve T cells as compared with memory/activated T cells. These observations are supported by a recent study showing that

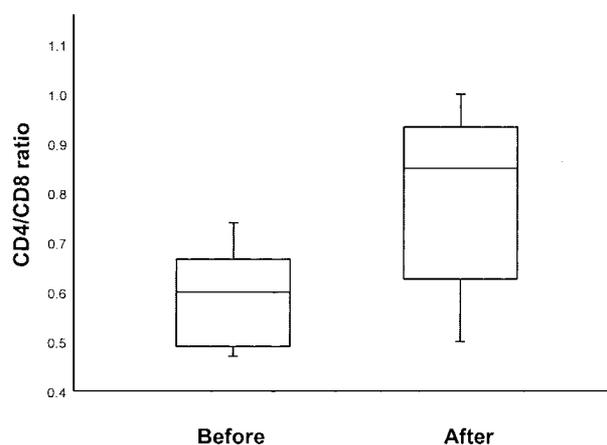


Fig. 6 Enhanced CD4:CD8 ratios as a result of vitamin E treatment. The CD4:CD8 ratios of the five patients with CD4:CD8 ratios below the reference values 0.8–3.4, are plotted. Boxes, 95% of the values; horizontal lines, medians; bars, SD; $P < 0.05$.

vitamin E supplementation to old mice leads to increased IL-2 production only in naïve T cells (39).

The mechanisms behind the selective targeting of Th1 cytokine production and naïve T cells by vitamin E remain elusive. Hydrogen peroxide was reported to inhibit specifically the activity of memory/activated T cells (30). Furthermore, it was shown that IL-10 production was particularly sensitive to oxidative stress. If this would occur also in patients with colorectal cancer, a more pronounced stimulatory effect on the memory/activated T cell subset and on IL-10 production would be expected as a result of the scavenging properties of vitamin E. Because this was not the case, the positive effect of vitamin E on Th1 cytokine production by naïve T cells, described here, may involve other mechanisms, including the previously described inhibition of prostaglandin production (32, 44) or via a direct stimulatory effect on T cell function (39, 48). In the study of Adolfsson *et al.* (39), it is suggested that the direct effect of vitamin E on T cells is a result of the enhanced IL-2 production in naïve T cells leading to proliferation and maturation of surrounding cells via an auto-paracrine loop.

The significance of IL-2 for cellular immunity is evident by its role in mediating clonal expansion of activated T cells (49). The induction of IL-2 production by activated T cells results in the up-regulation of high-affinity IL-2 receptors on T cells and, thus, in the responsiveness to IL-2 and progression through the cell cycle, as well as the differentiation of naïve T cells into effectors. IL-2 alone or in combination with other cytokines has, in various protocols against murine and human tumors, resulted in objective tumor regression and even in complete responses in a few patients (reviewed in Ref. 50). The importance of IFN- γ in relation to tumor immunity has been clearly demonstrated in STAT1 and IFN- γ -receptor knockout mice. Mice that are deficient in IFN- γ signaling components develop carcinogen-induced sarcomas at a higher frequency than do wild-type mice (51). Furthermore, the antitumor immunity is dependent on CD8⁺ T cells, which produce high amounts of IFN- γ but no IL-4 and/or IL-5 (Tc1 cells; Ref. 52). Tc1 cells, but not cells

producing high amounts of IL-4 and/or IL-5 and low amounts of IFN- γ (Tc2 cells), were capable of protecting against tumor challenges when adoptively transferred to syngeneic mice. These studies, together, support an active role of the immune system against tumors and suggest that IFN- γ is one of the most important components in this process, in part by modulating the immunogenicity of the tumor cells (53).

Vitamin E supplementation was also shown to increase CD4:CD8 ratios. It was previously reported that colorectal cancer patients with advanced disease have reduced numbers of CD4⁺ T cells leading to a decreased CD4:CD8 ratio (12). In our study 5 of 12 patients had CD4:CD8 ratios below the reference interval. Analysis of these patients revealed that vitamin E resulted in statistically significant increases and almost normalized CD4:CD8 ratios (Fig. 6; $P < 0.05$). CD4:CD8 ratios were, however, also increased significantly in patients with normal ratios before treatment, which indicated a generalized recruitment of T helper cells.

Apart from changes in CD4:CD8 ratios, no significant changes in subsets expressing other phenotypic markers, including CD62L, HLA-DR, and CD25, were observed (Data not shown). Thus, the stimulatory effect of vitamin E on Th1 cytokine production was not a result of an altered number of regulatory T cells (CD4⁺, CD25⁺), known to suppress T-cell function (54).

Most work on the positive effects of vitamin E on T-cell functions have been focused on the reversal of immune senescence in aging humans (32, 42). We found no correlation between age and increased IL-2 production in our trial, which included patients from 47 to 74 years of age (median 66, years). Thus, our data demonstrates that Th1 cytokine production can be enhanced by vitamin E in patients with colorectal cancer regardless of their age. It remains to be seen whether these effects would also sustain over longer time intervals of vitamin E supplementation, as would be necessary if combined with other immune interventions, such as specific vaccination with tumor antigens.

In closing, our results suggest that enhancing lymphocyte functions by dietary supplementation with vitamin E could precede more specific immune interventions, which may be dependent on a functional Th1 response. Finally, increased Th1 responses and, thus, enhanced antitumor activity by the immune system may be one molecular explanation for a beneficial role of vitamin E as primary prevention against cancer.

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