Effect of 50- and 100-mg vitamin E supplements on cellular immune function in noninstitutionalized elderly persons[1–3]

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ABSTRACT

Background: It has been suggested that vitamin E can counteract the age-associated decline in cellular immune responsiveness (CIR). Particularly, T helper cell type 1 (Th1) activity, ie, interferon (IFN) γ–producing Th1 activity and, hence, delayed-type hypersensitivity (DTH) would be enhanced by vitamin E supplementation.

Objective: Our aim was to study the effects of 6 mo supplementation with 50 and 100 mg vitamin E on CIR in the elderly.

Design: A double-blind, placebo-controlled trial was conducted in 161 healthy elderly subjects aged 65–80 y. CIR was measured in vivo by means of DTH skin tests and in vitro by assessing the production of interleukin (IL) 2, IFN-γ (a typical Th1 cytokine), and IL-4 (a typical Th2 cytokine) by peripheral blood mononuclear cells after stimulation with phytohemagglutinin.

Results: Both DTH and IL-2 production showed a trend toward increased responsiveness with increasing dose of vitamin E. However, IFN-γ production decreased whereas IL-4 production increased in the groups receiving vitamin E. Only the change in the number of positive DTH reactions was borderline significantly larger in the 100-mg vitamin E group than in the placebo group ($P = 0.06$, Bonferroni adjusted). Subjects receiving 100 mg vitamin E with low baseline DTH reactivity or who were physically less active had a significantly larger increase in the cumulative diameter of the skin induration resulting from the DTH test than did the placebo group ($P = 0.03$), although this difference was not significant after Bonferroni correction ($P = 0.07$).

Conclusion: Possible beneficial effects of 100-mg vitamin E supplementation may be more pronounced in particular subgroups of elderly subjects. *Am J Clin Nutr* 1999;69:1273–81.

KEY WORDS Elderly, vitamin E, cellular immune response, delayed-type hypersensitivity, T helper cells, cytokines

INTRODUCTION

It is well known that immune responsiveness declines with age (1). T cell–dependent functions are especially compromised, which is generally ascribed to the physiologic thymic involution that starts at an early age (2, 3). The age-related impaired T cell–dependent immune functions include defects in T cell–proliferative responses to mitogens, antibody response after primary immunization with T cell–dependent antigens, and particularly delayed-type hypersensitivity (DTH) and interleukin (IL) 2 production (2, 4–6). However, the production of other cytokines such as IL-4 and IL-6 seems to be elevated in older persons (4, 7). It would appear, therefore, that a shift from the proinflammatory type 1 T helper cell (Th1) response to the antiinflammatory type 2 (Th2) response is associated with aging (8, 9).

The decline in the cellular immune responsiveness in the elderly is generally associated with an increased incidence of infectious, neoplastic, and autoimmune diseases (3, 10). Therefore, an effective method to prevent or delay this age-related impairment of protective immune function would be of great public health significance.

Studies in both animals and humans indicate that adequate nutrition is necessary for optimal immune function (11). Supplementation with antioxidant vitamins especially has been associated with an enhancement of immune function in various animal models (12). More specifically for vitamin E, it has been shown that supplementation with a megadose (greater than the recommended dietary allowance) has a stimulatory effect on humoral and cell-mediated immunity (13). Because the depressed immune responsiveness observed in the elderly has been associated with an increase in free radical formation (14), the need for vitamin E in old age to maintain optimal immune function might be higher than the current Dutch recommended dietary allowance (15).

The mechanism for the immune-enhancing effect of vitamin E has not yet been completely elucidated. Presumed mechanisms for this effect include both a direct and an indirect effect (through macrophages) of vitamin E on T cell functioning (16). The direct effect of vitamin E might be mediated through vitamin E–induced changes in the T cell membrane receptor molecules that are involved in the immune response (14). Through its

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[2] Supported by grants from the Dutch Praeventiefonds and F Hoffmann-La Roche, Ltd.

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Received December 2, 1997.

Accepted for publication November 19, 1998.

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antioxidant function, vitamin E could also decrease the production of immunosuppressive factors such as prostaglandin E_2 (PGE_2) and hydrogen peroxide by activated macrophages (14, 16). It was reported that PGE_2, apart from being immunosuppressive, regulates the balance of activity between Th1 and Th2 subsets in favor of the latter (17). Thus, it might be speculated that through its action on PGE_2 synthesis, vitamin E stimulates Th1-like immune responses (18).

Clinical trials in elderly subjects that studied the effect of vitamin E supplementation on immune responsiveness are scarce and the results inconsistent (19–24). Positive results have been found by Meydani et al (21), who showed in 2 trials conducted in healthy American elderly a stimulatory effect on variables related to T cell–dependent immune responsiveness after 1 mo of supplementation with 800 mg vitamin E and after 4.5 mo supplementation with 60, 200, or 800 mg vitamin E (23). From the latter study the authors concluded that 200 mg would be the optimal dose of vitamin E for the immune response in elderly subjects with initial serum vitamin E concentrations ≤27.9 μmol/L.

We investigated whether a 3- or 6-mo (12- or 24-wk) supplementation with lower doses of vitamin E (50 or 100 mg/d) in a healthy Dutch elderly population would also be effective in enhancing cellular immune response. T cell reactivity was assessed with both in vivo DTH tests with recall antigens and in vitro (mitogen-induced IL-2 production) test methods. To assess the effect of vitamin E on the balance of type 1 and type 2 cytokines, which has never been studied in humans before, we also measured interferon (IFN) γ and IL-4 in the supernates of mitogen-stimulated T cell cultures.

SUBJECTS AND METHODS

Study subjects

The 12 general practitioners of the city of Wageningen, Netherlands, selected 1079 independently living elderly subjects aged 65–80 y and asked them to participate in the trial. Selection was based on the following inclusion criteria: no diseases or treatments known to have an effect on the immune response (rheumatoid arthritis, Crohn disease, colitis ulcerosa, multiple sclerosis, active tuberculosis, major surgery, radiotherapy, chemotherapy, or corticosteroid treatment), no diseases that might interfere with the metabolism of vitamin E (liver insufficiency, diabetes mellitus, or fat malabsorption syndrome), no coagulation disorder or use of anticoagulants that interfere with vitamin K metabolism (acenocoumarol and fenprocoumon), no use of antioxidative medication (*N*-acetylcysteine), and no disease or condition that would interfere with the ability to participate (sensory deficit, physical handicap, being bedridden, having a mental disorder, or terminal disease).

Four hundred twenty-eight subjects did not respond, 260 refused, and 391 were willing to participate. The volunteers were sent a questionnaire in which the above-mentioned inclusion criteria were checked and some additional questions were asked about health status and use of supplements. From this group, another 187 subjects were excluded because they did not meet the above-mentioned criteria (n = 60) or because they regularly used a vitamin-mineral supplement or a supplement possibly containing vitamins, such as fish oil and garlic supplements (n = 127). From the 204 eligible subjects, 84 men and 84 women were randomly selected for the trial. Just before the start of the trial, 7 subjects had second thoughts and withdrew, leaving a study population of 161 eligible subjects, 82 men and 79 women. Informed consent was obtained from all of these subjects before any trial-specific procedures were performed. The study protocol was approved by the Medical Ethics Committee of the Agricultural University Wageningen.

Study design and procedures

The study was designed as a placebo-controlled, double-blind, randomized, parallel group trial. The supplement consisted of 100 mg DL-α-tocopheryl acetate, 50 mg DL-α-tocopheryl acetate, or placebo capsule containing 155 mg soybean oil (all from F Hoffmann-La Roche Ltd, Basel, Switzerland) that looked identical to the other supplements. Per sex stratum, the 3 supplements were randomly assigned in such a way that equal numbers of subjects were assigned each supplement at the enrollment of every sixth subject (ie, 2 in each group; balanced randomization) to account for seasonal effects.

At baseline (September 1996) and after 12 and 24 wk of supplementation, DTH skin tests were administered and evaluated. Before and after the 24-wk intervention period, nonfasting blood samples were taken for the evaluation of cell-mediated immunity in vitro. These blood samples were also used to measure antioxidant vitamin status. In addition, body weight and height were determined on both occasions, and at the initial visit the subjects also completed a questionnaire on concomitant diseases (ie, diseases that did not lead to exclusion), use of medication, and smoking habits. Physical activity was assessed with the Physical Activity Scale for the Elderly (PASE) questionnaire developed by The New England Research Institute (25) and translated and adjusted for Dutch elderly subjects by our department (26). Halfway through the intervention period and at the end of the study, compliance was assessed by pill counts. During the entire intervention period, the subjects were asked to mark on a calendar any intercurrent diseases or medicine use as well as the acute onset of any of the following symptoms of an acute upper respiratory tract infection (URTI): cough, rhinitis, sore throat, rigor or chills, fever, myalgia, malaise, and headache. Any combination of ≥2 of these symptoms, including ≥1 of the first 3 mentioned symptoms that lasted for ≥2 d was considered a URTI.

Assessment of delayed-type hypersensitivity

To assess cutaneous DTH, the Multitest CMI (Institut Mérieux Transplant BV, Amstelveen, Netherlands) was used. The Multitest CMI is an applicator consisting of 8 heads preloaded with 7 recall antigens: tetanus toxoid, diphtheria toxoid, streptococcus (group C), tuberculin, *Candida albicans*, *Trichophyton mentagrophytes*, *Proteus mirabilis*, and one glycerin control.

The test was administered on the volar surface of the forearm and evaluated after 48 h by the same nurse. Subsequent tests were administered on the same arm or on the other arm. The circumferences of the skin indurations were marked with a ballpoint pen and copied on tape. The size of each induration was measured in 2 (perpendicular) directions, both measured in duplicate. The mean of the 4 readings was used. According to the manufacturer's instruction, a reaction was considered positive when the mean diameter was ≥2 mm. Two DTH scores were calculated: *1*) the number of positive reactions and *2*) their cumulative diameter, ie, the sum of the diameters of all positive reactions.

Blood collection and preparation

Venous blood was obtained by using four 10-mL (Omnilabo, Breda, Netherlands) and one 7-mL Vacutainers (Becton Dickin-

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son, Meylan-Cedex, France) containing heparin. The blood samples were collected between 0900 and 1230 and the subjects were asked to abstain from consuming any fruit juice or a hot meal before venipuncture to avoid an acute effect on the antioxidant and cholesterol plasma concentrations.

The 7-mL evacuated tube was used to separate the plasma, in which the antioxidant vitamins were measured. Centrifugation was done at room temperature for 10 min at 1000 × *g*. Plasma for determination of vitamin C (0.5 mL) was stabilized within 1 h after blood collection with 4.5 mL 5% metaphosphoric acid solution. All plasma samples were stored at −80 °C.

Peripheral blood mononuclear cells (PBMCs) were isolated from the four 10-mL evacuated tubes by density gradient centrifugation with Lymphoprep (Life Technologies, Breda, Netherlands) within 2 h after venipuncture. The cells at the plasma-Lymphoprep interface were harvested, washed twice in complete RPMI medium (RPMI/HEPES; Boehringer/Biowhittaker, Verviers, Belgium) with 10% (by vol) heat-inactivated fetal calf serum (Integro, Zaandam, Netherlands), and penicillin and streptomycin (100 000 U/L and 0.1 g/L, respectively), both from Sigma Chemical Company, St Louis. The cells were resuspended at 10×10^9/L in complete RPMI mixed 1:1 with 10% dimethyl sulfoxide (hybrimax; Sigma Chemical Company) and distributed in cryovials, which were kept overnight at −80 °C. The next morning the vials were transferred to liquid nitrogen (−196 °C), where they were stored until analyzed.

Plasma antioxidant vitamin concentration

Within 3 mo after blood collection, the plasma samples were analyzed for α-tocopherol, β-carotene, and vitamin C in the laboratories of F Hoffmann-La Roche Ltd (Basel, Switzerland) by means of reversed-phase HPLC (α-tocopherol and β-carotene) and fluorometry (vitamin C) as described by Hess et al (27) and Brubacher and Vuilleumier (28), respectively.

In vitro interleukin production

IL-2, IFN-γ, and IL-4 production by PBMCs pre- and postintervention were measured simultaneously after 40 h of stimulation with optimal (3 mg/L) and suboptimal (1 mg/L) concentrations of purified phytohemagglutinin (PHA) (HA 16; Murex Diagnostics Benelux BV, Breukelen, Netherlands). To this end, patient and control PBMCs were thawed and washed twice with complete RPMI medium and 2.5×10^6 cells were cultured in 1 mL complete RPMI containing 0, 1, or 3 mg PHA/L in 24-well culture plates (Costar, Cambridge, MA). Viability of the cells, as evaluated by trypan blue exclusion, always exceeded 90%.

To avoid intertest variation, pre- and postintervention samples from each individual were cultured on the same plate. In addition, control PBMCs from a large batch of cryopreserved PBMCs were included on every plate to allow for evaluation of intratest variation. CV values ranged from 13% for 1 mg PHA/L–induced IL-2 production to 31% for IFN-γ production.

After incubation (40 h at 37 °C in 5% CO_2), the plates were centrifuged for 10 min at 650 × *g*. Supernates were collected and stored at −80 °C until assayed. Cytokine production was measured by a standardized enzyme-linked immunosorbent assay (ELISA) using commercial kits (IL-2, IFN-γ: Medgenix, Fleurs, Belgium; screening line, IL-4: CLB, Amsterdam). In each ELISA plate a standard curve was produced with recombinant IL-2, IFN-γ, or IL-4 provided by the supplier. Each supernate was tested at 2 dilutions to be certain that at least one reading could be read at the standard curve (1:3 and 1:10 for IL-2, and 1:1 and 1:3 for IL-4 and IFN-γ). Optical density readings obtained at these 2 dilutions were converted to concentrations in the undiluted supernate by direct interpolation on the standard curve and accounting for the supernate dilution factor. When both dilutions could be read at the linear part of the standard curve, the mean of the 2 outcomes was used. If the 2 outcomes varied much, which was seldom the case, or if the results could not be read, a new ELISA was done with other dilutions.

Statistical analysis

The number of subjects needed per group (n = 44) was based on an estimated expected difference between the changes in the cumulative DTH score in the vitamin E and placebo groups of 25% (estimated SD of percentage change = 40) with $\alpha = 0.05$ and $\beta = 0.10$. This is a conservative estimate of the effect of 50- and 100-mg vitamin E given the mean increase of 52.8% that was observed in the group supplemented with 800 mg vitamin E in the study of Meydani et al (21). Because recruitment was successful and to take into account some loss to follow up, at the start of the study each group had ≥53 subjects (total n = 161).

Only 2 female subjects, both from the placebo group, dropped out during the intervention period: 1 because of supplement intolerance and the other because of surgery. All of the remaining 159 subjects gave blood and were tested for DTH both before and after the intervention period. However, 2 other people—a married couple—were excluded from the analyses because we suspected that they had inadvertently switched their capsules (containing 0 and 100 mg vitamin E). Compliance according to pill counts was excellent. All subjects took ≥83% of the pills; in fact, only 5 subjects took <90% of the pills. Results are reported for 157 subjects unless stated otherwise.

Means and SDs or percentages of the baseline characteristics were calculated for the 3 supplementation groups separately and then inspected for possible relevant differences, so as to judge whether inclusion as a covariable in the multiple analysis would be indicated. Simple regression analysis with dose of vitamin E as the independent variable and change in plasma vitamin E concentration as the dependent variable was used to test whether there was a significant increasing trend in plasma vitamin E concentration with increasing dose (trend test). To test whether there was a concomitant significant change in plasma β-carotene and vitamin C within the 3 supplementation groups after the 24-wk intervention period, paired Student's t tests were used. Then, analysis of variance (ANOVA) was used to test whether these within-group changes were different between the groups.

Spearman correlation was used to study the relation between intraindividual changes in plasma concentrations of vitamin E and changes in the immune variables. To test whether the within-group changes in the immune variables differed from zero, a paired t test was performed for cumulative DTH score and Wilcoxon's signed-rank test was performed for the number of positive DTH responses and the cytokine production capacity.

To test whether the changes in cumulative DTH score in the 50- and 100-mg vitamin E–supplemented groups differed from the change in the placebo group, the differences were evaluated in a regression model by using 2 indicator variables for the 3 groups. The within-group changes in the other immune variables were compared by using Wilcoxon's rank-sum test. Because more than one comparison was made, we also performed Bonferroni corrections to have a more conservative estimate of

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TABLE 1
Baseline characteristics

Characteristic	Placebo group (n = 26 M, 24 F)	Vitamin E group	
		50 mg (n = 27 M, 27 F)	100 mg (n = 28 M, 25 F)
Age (y)	71.1 ± 3.5[1]	70.5 ± 3.9	71.5 ± 4.0
Body mass index (kg/m^2)	26.6 ± 3.1	26.8 ± 3.3	27.5 ± 3.6
Smokers (%)	18.0	16.7	11.3
Perceived health score[2]	7.9 ± 0.9	7.7 ± 0.7	7.5 ± 1.0
Have at least a "higher vocational education" (%)	30.0	27.8	28.3
PASE score[3]	114.0 ± 41.9	101.9 ± 38.3	112.8 ± 43.7

[1] $\bar{x}$ ± SD.
[2] Self-rated health score on a scale from 1 (very bad) to 10 (excellent).
[3] Activity score based on the self-administered Physical Activity Scale for the Elderly questionnaire in which physical activities of the past week are assessed; range in PASE score in the total group: 25.0–231.2.

the P value. For the cumulative DTH score, repeated-measures ANOVA was used to test the effect of time and dose and to test whether there was an interaction between these 2 effects.

Multiple regression analysis with change in the immunologic variables as the dependent variable and group (again, 2 indicator variables) and baseline value of the immune variables as well as baseline or change in plasma concentration of the antioxidant vitamins as the independent variables was used to estimate the effect of the supplementation adjusted for the variables mentioned. Least-squares means were computed to obtain an adjusted value for the mean change in DTH score. All tests were conducted at the 5% significance level. All data analyses were performed by using the SAS 6.09 statistical package (29).

RESULTS

Baseline characteristics and plasma antioxidant vitamin concentrations

The baseline characteristics of the 3 supplementation groups are shown in **Table 1**. The groups appeared to be quite similar as a result of the randomization procedure. Therefore, we decided not to include any of these variables in the multiple analysis.

In **Table 2** the baseline plasma concentrations of vitamin E and the other antioxidant vitamins and provitamins are presented as well as the change after 24 wk. At baseline, the plasma α-tocopherol and vitamin C concentrations were slightly higher in the group receiving 100 mg vitamin E. There was a significant trend toward increased postintervention vitamin E plasma concentrations with increasing dose of vitamin E (P = 0.0001). Although there were also some significant changes, albeit small, in the plasma concentrations of β-carotene and vitamin C within the supplementation groups, these changes did not differ significantly among the 3 groups (one-way ANOVA).

Delayed-type hypersensitivity

At baseline, the frequency of positive reactions to the individual antigens in the total study population was 45.9% for tetanus, 33.1% for diphtheria, 5.7% for streptococcus, 47.1% for tuberculin, 28.0% for *Candida*, 12.7% for *Trichophyton*, and 61.8% for *Proteus*. These frequencies were not significantly different among the 3 groups. Partial correlation (correlation adjusted for sex and age) between the plasma concentration of vitamin E and the 2 DTH scores at baseline was low (r = 0.13, P = 0.10 for the number of positive reactions and r = 0.11, P = 0.19 for cumulative DTH score).

The baseline DTH scores and the change in these scores after 12 and 24 wk of intervention are given in **Table 3**. These data show a significant increasing trend in DTH scores with time (P = 0.0001) and a similar but nonsignificant trend with dose of vitamin E after 24 wk of intervention (P = 0.58). The effect of time was not different for the 3 different doses (repeated-measures ANOVA). With use of Wilcoxon's rank-sum test, the increase in the number of positive reactions after 24 wk was borderline significantly greater in the 100-mg vitamin E group than in the placebo group (P = 0.03, Bonferroni adjusted: P = 0.06).

TABLE 2
Baseline and change in plasma concentrations of antioxidant vitamins after 24 wk of intervention[1]

Plasma antioxidant vitamin	Placebo group (n = 50)		Vitamin E group[2]			
			50 mg (n = 54)		100 mg (n = 52)[3]	
	Baseline	Change	Baseline	Change	Baseline	Change
α-Tocopherol (μmol/L)	29.4 ± 6.9	0.4 ± 3.6	28.8 ± 6.8	10.1 ± 5.0[4]	31.1 ± 6.0	15.8 ± 7.4[4]
α-Tocopherol:cholesterol (mmol/mol)	5.0 ± 0.9	0.0 ± 0.5	5.0 ± 0.0	1.6 ± 0.7[4]	5.1 ± 0.9	2.2 ± 0.9[4]
β-Carotene (μmol/L)	0.5 ± 0.3	0.0 ± 0.2	0.6 ± 0.4	−0.1 ± 0.3[5]	0.5 ± 0.3	−0.1 ± 0.1[4]
Ascorbic acid (μmol/L)[6]	49.8 ± 20.8	5.5 ± 16.8[5]	54.1 ± 19.0	6.8 ± 19.4[5]	57.7 ± 21.8	3.9 ± 17.7

[1] $\bar{x}$ ± SD.
[2] Significant dose response for α-tocopherol and α-tocopherol:cholesterol, P = 0.0001 (trend test).
[3] Blood could not be drawn from one subject during the postintervention measurements.
[4,5] Significantly different from zero (paired Student's t test): [4] P = 0.0001, [5] P < 0.05.
[6] n = 48 for the placebo group; n = 50 for the 100-mg vitamin E group.

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TABLE 3
Baseline and change in delayed-type hypersensitivity (DTH) scores after 12 (change 1) and 24 (change 2) wk of intervention[1]

Group and DTH measure	Baseline	Change 1	Change 2
Placebo (n = 49)[2]			
Cumulative DTH score (mm)	15.0 (7.0, 20.1)	1.0 ± 8.6	4.2 ± 7.3[3]
Positive reactions (n)	2.0 (2.0, 4.0)	−0.2 ± 1.2	0.3 ± 1.2
Vitamin E			
50 mg (n = 54)			
Cumulative DTH score (mm)	11.5 (6.9, 17.4)	2.7 ± 7.0[4]	4.6 ± 7.8[3]
Positive reactions (n)	2.0 (1.0, 3.0)	0.0 ± 1.3	0.4 ± 1.5[4]
100 mg (n = 53)			
Cumulative DTH score (mm)	12.7 (6.4, 19.1)	2.2 ± 8.3	6.0 ± 9.3[3]
Positive reactions (n)	2.0 (1.0, 3.0)	0.2 ± 1.2	0.8 ± 1.5[4,5]

[1] Baseline: median (lower, upper quartile); changes: $\bar{x}$ ± SD.
[2] The DTH test could not be read in one subject after 24 wk.
[3,4] Significantly different from zero (paired t test or Wilcoxon signed-rank test): [3] $P < 0.001$, [4] $P < 0.05$.
[5] Significantly different from placebo group, $P = 0.03$; with Bonferroni adjustment, $P = 0.06$ (Wilcoxon's rank-sum test).

This difference in changes also remained borderline significant after adjustment for the baseline DTH score and plasma antioxidant concentrations or changes in these concentrations. On the other hand, the increase in cumulative DTH score in both vitamin E groups did not differ significantly from the increase in the placebo group. Adjustment for the above-mentioned variables did not materially alter this finding. In the total study population, no correlation was found between postintervention plasma vitamin E concentration or change in plasma vitamin E concentration and change in any of the DTH variables.

To avoid distortion by concomitant chronic diseases or infectious diseases occurring during intervention, analyses were also carried out in a healthy, so-called "senieur" population that was selected according to the SENIEUR protocol of the working party of the EURAGE Concerted Action Programme on Ageing of the European Community (30). For that purpose, we excluded all people who reported having cancer (past or present), had any chronic inflammation or skin disease, or had surgery within 12 wk before DTH measurement. In addition, those who reported >2 symptoms of acute URTI for 2 d or had any other infection diagnosed by their general practitioner (mainly cystitis) in the period from, respectively, 2 and 4 wk before until 1 wk after the DTH test was performed were also excluded.

In this senieur population (placebo group: n = 31; 50-mg vitamin E group: n = 35; 100-mg vitamin E group: n = 36), the increases in cumulative DTH score after the 24-wk intervention were not significantly greater in the vitamin E–supplemented groups than in the placebo group, nor were the increases greater for the number of positive reactions.

To see whether an effect of vitamin E was stronger in people who were (possibly) in a less healthy condition, we repeated the analyses in subgroups of people who at baseline rated their own health as ≤7 (on a scale of 1, very bad, to 10, excellent; n = 62), had a suboptimal plasma α-tocopherol concentration (<30 μmol/L; n = 78), were less physically active (lower half of the PASE score, "low activity group"; n = 75), or were relatively anergic (not more than 2 positive DTH reactions, "low DTH group"; n = 88). In all of these subgroups, the number of people in each supplementation group was about the same. In both the low DTH group and the low activity group, we found a significantly larger increase in both DTH scores after 24 wk in the 100-mg vitamin E group than in the placebo group with and without adjustment for baseline DTH score and antioxidant vitamin concentrations ($P < 0.05$). However, after adjustment for multiple comparisons by using Bonferroni correction, the differences were no longer significant. The adjusted mean changes in cumulative DTH score and number of positive DTH responses in the low DTH group are shown in **Figures 1** and **2**. The adjusted mean (±SEM) changes in the low activity group after 24 wk were 1.6 ± 1.8, 4.7 ± 1.5, and 7.2 ± 1.7 mm for cumulative DTH score and −0.2 ± 0.3, 0.3 ± 0.2, and 0.9 ± 0.2 for number of positive reactions in the 0, 50-, and 100-mg vitamin E groups, respectively.

The low DTH and low activity subgroups did not differ significantly from the total study population for most variables measured in this study, including age, body mass index, and baseline and changes in plasma antioxidant vitamin concentrations. Only the low activity group had—as was expected—a significantly higher percentage of women than did the total study population (61% compared with 49%), a lower (NS) percentage of smokers (9.3% compared with 15.3%), and a slightly lower (NS) baseline cumulative DTH score ($\bar{x}$ ± SEM: 12.1 ± 0.9 compared with

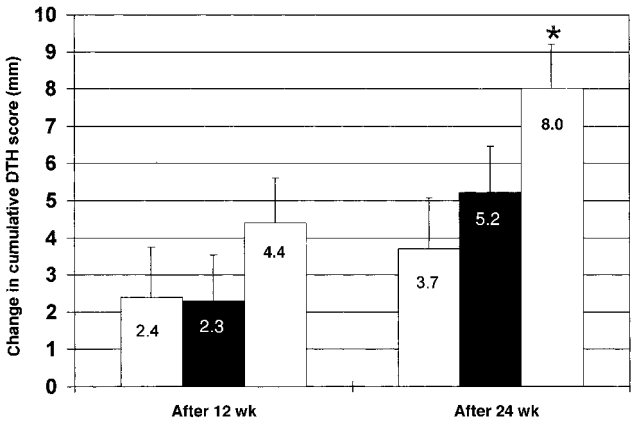

FIGURE 1. Mean, adjusted [for baseline delayed-type hypersensitivity (DTH) score and baseline plasma antioxidant vitamin concentration] change in cumulative DTH score after 12 and 24 wk of vitamin E supplementation (□, 0 mg, n = 27; ■, 50 mg, n = 29; ▩, 100 mg, n = 32) in elderly subjects with ≤2 positive DTH reactions at baseline. *Significantly different from placebo, P = 0.03; with Bonferroni adjustment, P = 0.07 (F test).

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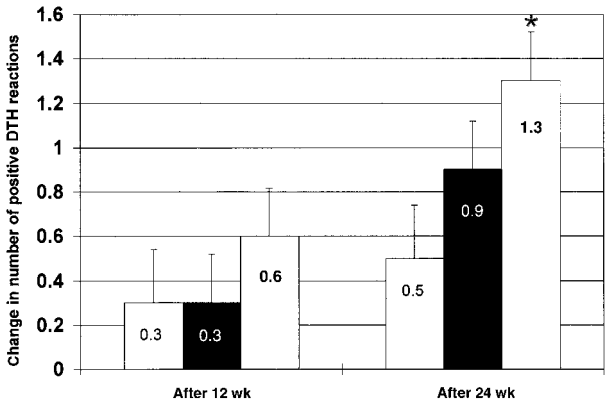

FIGURE 2. Mean, adjusted [for baseline delayed-type hypersensitivity (DTH) score and baseline plasma antioxidant vitamin concentration] change in number of positive DTH reactions after 12 and 24 wk of vitamin E supplementation (□, 0 mg, $n = 27$; ■, 50 mg, $n = 29$; ▩, 100 mg, $n = 32$) in elderly subjects with ≤2 positive DTH reactions at baseline. *Significantly different from placebo, $P = 0.04$; with Bonferroni adjustment, $P = 0.08$ (F test).

13.5 ± 0.7 mm). The members of the low activity group were not the same as those of the low DTH group because the overlap between these 2 groups was not larger than expected. For example, the percentage of people in the low activity group with a low DTH score was 60%; in the total study population it was 57%. As was expected, in the complementary subgroups (people with PASE scores > 111 and people with > 2 positive DTH reactions at baseline) the increases in DTH scores over time were not different between the 3 study groups, but may have been somewhat smaller in the vitamin E–supplemented groups.

Cytokine production

At baseline there was a small but significant correlation between plasma vitamin E concentration and PHA (3 mg/L)-stimulated IL-4 production after adjustment for sex, age, and plasma vitamin C and β-carotene concentrations (partial $r = 0.18$, $P = 0.04$). A small negative correlation (NS) existed between plasma vitamin E concentration and IFN-γ production after stimulation with 3 mg PHA/L (partial $r = -0.14$, $P < 0.10$).

The effect of vitamin E supplementation on the production capacity of IL-2, IFN-γ, and IL-4 by PBMCs is shown in **Table 4**. Only the PHA (1 mg/L)-stimulated production of IL-2 was significantly increased after 24 wk ($P < 0.01$). However, the increase in IL-2 was not significantly greater in the vitamin E groups than in the placebo group. In contrast, the production of IFN-γ was reduced after the intervention period. After stimulation with 1 mg PHA/L, the decrease in the IFN-γ production in the 50-mg vitamin E group was slightly larger than that in the placebo group ($P = 0.04$; Bonferroni adjusted: $P = 0.07$). In both vitamin E groups, IL-4 increased significantly over time after stimulation with 1 mg PHA/L. For IL-2, however, these changes did not differ significantly from the change in the placebo group.

The ratio of IFN-γ to IL-4 production can be used as a measure of the Th1-Th2 subset activity balance. Essentially identical results were obtained when these ratios were compared. At 1 mg PHA/L, the median ratio tended to decrease from 2.85 to 2.29 in the placebo group, whereas significant decreases from 2.94 to 1.38 and from 3.07 to 1.81 were noted in the 50- ($P = 0.0001$) and 100-mg vitamin E ($P = 0.01$) groups, respectively. However, these significant decreases in the vitamin E groups were not significantly different from the decrease in the placebo group. For cell supernates treated with 3 mg PHA/L, there were similar but less pronounced trends and no significant changes were noted.

Because the log of the ratio of post- and preintervention cytokine production was normally distributed in contrast with the absolute change, as presented in **Table 4**, the former was used in a multiple regression model to adjust for baseline differences in cytokine production and plasma antioxidant vitamin concentrations. Adjustments for these variables did not alter the results markedly.

Subgroup analysis, as outlined above for DTH evaluations, did not yield any result that differed significantly from that in the total study population. Nor was there a correlation between postintervention or change in vitamin E plasma concentration and change in the production of any of the cytokines.

DISCUSSION

In this trial in which we studied the effect of 50- and 100-mg vitamin E supplements on several indexes of the cellular immune

TABLE 4
Baseline and change in phytohemagglutinin (PHA)-stimulated cytokine production by peripheral blood mononuclear cells (PBMCs) after 24-wk intervention[1]

Cytokine and PHA concentration	Placebo group ($n = 44$)		Vitamin E group			
			50 mg ($n = 50$)		100 mg ($n = 45$)	
	Baseline	Change	Baseline	Change	Baseline	Change
IL-2 (ng/L)						
1 mg PHA/L	495 (157, 1503)	421[2] (−125, 1172)	467 (65, 1664)	355[2] (0, 1003)	662 (195, 1722)	420[2] (−72, 861)
3 mg PHA/L	2780 (1820, 6499)	122 (−1874, 1479)	2496 (1241, 5394)	187 (−863, 1242)	2989 (1554, 4788)	262 (−514, 1208)
IFN-γ (10^3 U/L)						
1 mg PHA/L	37 (13, 91)	1 (−15, 20)	37 (18, 89)	−10[2,3] (−27, 0)	37 (18, 71)	−8 (−33, 8)
3 mg PHA/L	44 (28, 182)	−4 (−68, 8)	75 (37, 150)	−17[4] (−55, 13)	67 (34, 143)	−15[4] (−78, 14)
IL-4 (ng/L)						
1 mg PHA/L	13 (8, 26)	2 (−4, 8)	12 (7, 26)	2.5[4] (−2, 9)	10 (6, 22)	3[2] (0, 10)
3 mg PHA/L	35 (19, 64)	−1 (−6, 5)	32 (21, 76)	−1 (−20, 13)	36 (17, 62)	1 (−7, 14)

[1] Median (lower, upper quartile). IL, interleukin; IFN, interferon. Enough PBMCs could not be isolated for all subjects so group sizes are somewhat reduced; data are missing for some measurements.

[2,4] Significantly different from 0 (Wilcoxon's signed-rank test): [2] $P < 0.01$, [4] $P < 0.05$.

[3] Significantly different from change in placebo group, $P = 0.04$; with Bonferroni adjustment, $P = 0.07$ (Wilcoxon's rank-sum test).

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response, we found that elderly subjects who were apparently healthy but had a low DTH response or were less physically active at baseline had an enhanced DTH response after 6 mo supplementation with 100 mg vitamin E compared with placebo. However, this difference was not significant when adjusted for multiple comparisons. This difference was also present in the total study population but was less pronounced. No effect of 6 mo supplementation with 100 mg vitamin E was observed on Th1 and Th2 cytokine production by PBMCs.

Despite randomization, elderly subjects in the 100-mg vitamin E group had a higher mean plasma vitamin C concentration at baseline, whereas the mean baseline value of the other potential confounders such as plasma concentration of the other 2 antioxidant vitamins, PASE score, and immune variables differed only slightly between the groups. In addition, during the intervention period there was a significant decrease in the plasma β-carotene concentration in the vitamin E–supplemented groups, whereas vitamin C increased significantly in the 50-mg vitamin E and the placebo groups. This might be a consequence of regression to the mean because the baseline β-carotene and vitamin C plasma concentrations were both strongly correlated with the change in these concentrations (Spearman rank correlation: $r = -0.53$, $P = 0.0001$ for β-carotene; Pearson correlation: $r = -0.36$, $P = 0.0001$ for vitamin C). Finally, the percentage of people experiencing at least one URTI or having themselves vaccinated against influenza within a few weeks after the start of the intervention period was higher (NS) in the vitamin E–supplemented groups than in the placebo group: 52.8%, 61.1%, and 48.0% with at least one URTI and 52.8%, 50.0%, and 42.0% vaccinated in the 100-mg vitamin E, 50-mg vitamin E, and placebo groups, respectively. However, adjustment for the above-mentioned small differences between the supplemented groups did not influence the results appreciably. Selection and observation bias can be ruled out in this study because there was minimal loss to follow-up and because the study was double-blind and randomized.

Frazer et al (31) and Lesourd et al (32) reported high reproducibility for cumulative DTH scores, especially in people who remained healthy (32). In our study, the Multitest CMI was applied by the same nurse at the same time of the day before and after intervention. Despite this, the rank correlations between cumulative DTH scores and between the number of positive DTH reactions obtained before and after 24 wk in those elderly subjects in the placebo group who did not experience a URTI [DTH variations can be expected during infections (32)] were only 0.70 and 0.50, respectively. In fact, ≈25% of all subjects (with or without a URTI) had less positive reactions after the intervention period compared with baseline. Part of the explanation for this intraindividual variability is that the responsiveness depends on the pressure with which the Multitest is applied. According to Jensen et al (33), a low incidence of positive reactions, as was seen in our study, may be due in part to false-negative results when reactions are only read after 48 h instead of after both 24 and 48 h. If the vitamin E–supplemented groups reached peak reactions earlier, reading the DTH tests at 48 h might explain the observation that the difference in number of positive reactions was significant, whereas the difference in total diameter of induration was not. This explanation, however, does not seem very probable.

Because a high intertest variability is inherent to cytokine production tests, PBMCs collected pre- and postintervention were stored in liquid nitrogen and assayed simultaneously in the same run. However, although certainly not large for these types of tests, the remaining intratest variation was still considerable. Originally, the group size was based on a power calculation for the DTH parameters. But because of the large variation in the cytokine production assays and the smaller numbers per group, the power of the study was insufficient for differences to be observed in IL-2 response in this study.

This study was based mainly on the presumption that elderly subjects would have reduced immune responses, particularly diminished Th1 function. Unfortunately, however, this could not be verified for the DTH scores because there are no reference values for younger Dutch people. US elderly have much higher DTH scores than the nonelderly (34), but comparison is not very useful because the frequency of positive DTH responses depends on public immunization policies and the prevalence of infection in a community (31). When the balance of Th1 to Th2 cytokines in our study population was compared with that of another, younger population (our healthy laboratory staff, unpublished observations, 1998), the balance seemed indeed to be shifted to Th2.

Remarkable in this study was the significant increase in both the cumulative DTH score and the PHA (1 mg/L)-induced IL-2 production in the placebo group. Apart from a possible seasonal or placebo effect, this might have been because of the significantly increased plasma vitamin C concentration that was observed in the placebo group. In addition, the increase can at least partly be explained by the apparent stimulating effect of influenza vaccination and URTIs on these immune measures because both the increase in IL-2 and DTH score was slightly larger (NS) in the group of 76 elderly who had themselves vaccinated against influenza than in those not vaccinated. Also, the increase in DTH was slightly larger in the group that experienced a URTI than in the group that did not. This is in line with the observations of Lesourd et al (32), who reported a rebound effect that can occur after an immunodepressive period during infection. Another explanation for the observed increase in DTH might be a booster effect, although this effect has been reported to be minimal (32) or even absent when DTH is assessed by means of Multitest CMI (35). Although both DTH scores and IL-2 production increased, the increases in these variables were not correlated.

In 3 other trials, Meydani et al (21–23) observed a significant positive effect of vitamin E supplementation on DTH in healthy elderly subjects. In the last trial (23), which examined doses of 60, 200, and 800 mg vitamin E/d, the only dose for which an effect was found that was significantly different from that of the placebo group was 200 mg vitamin E. Significant positive effects on DTH were also found in 2 trials conducted by Bogden et al (36, 37) in which healthy elderly subjects were supplemented with a multivitamin-mineral supplement that also contained ≥20 mg vitamin E. In contrast with our present report, in these trials the significant beneficial effects of supplementation were not restricted to particular subgroups of elderly subjects.

The most likely explanation for the difference between our results and those of the studies of Meydani et al is the difference in vitamin E dose; Meydani et al did not test a dose of 100 mg, and we did not test a dose of 200 mg vitamin E. Other possible explanations might be the higher baseline plasma vitamin E concentration in our study or other differences between the study populations.

The fact that the differences between the increases in DTH after supplementation with 100 mg vitamin E for 6 mo were larger than those with the placebo in the low DTH and low activity subgroups, factors for which a relation with increased mortality has been reported (38, 39), might indicate that beneficial

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effects of vitamin E supplementation are more pronounced in elderly persons in a suboptimal state of health. However, we do not have other information to substantiate this speculation. Furthermore, all of the above-mentioned studies enrolled apparently healthy elderly subjects and in the studies of Meydani et al they were even screened by a general practioner. Still, one other study showing a positive effect of multivitamin supplementation on DTH was indeed conducted among institutionalized and therefore less fit elderly subjects (40).

We did not find a significant effect of vitamin E supplementation on IL-2 production in the total study population, nor in any subgroup. Meydani et al (21) and Chandra (41) reported significant effects of vitamin E and multivitamin-mineral supplementation, respectively, on in vitro IL-2 production in the elderly. Meydani et al, however, assessed IL-2 production after stimulation with the T cell mitogen concanavalin A, whereas we stimulated with PHA. In the same study they did not find an effect of vitamin E supplementation on PHA-induced lymphocyte proliferation, which suggests that the effect of vitamin E is specific. Furthermore, the vitamin E doses we used were much lower than those used in Meydani et al's study (800 mg vitamin E). Finally, in Chandra's study not only were other vitamin supplements given but the duration of the intervention was twice as long as in our study.

Little is known about vitamin E modulation of cytokine production. Because it was suggested that vitamin E, via a reduction of PGE_2 production, could stimulate a Th1-like response (18), we expected an increase in the production of the type 1 cytokines IL-2 and IFN-γ in the vitamin E–supplemented groups and a similar decrease in the production of the type 2 cytokine IL-4. In contrast, we found a significant decrease in IFN-γ production and a significant increase in IL-4 production in both vitamin E–supplemented groups. The decrease in IFN-γ was, however, not correlated with the increase in IL-4 production. In only 2 other studies in mice was the effect of vitamin E on the production of Th1 and Th2 cytokines determined. First, Wang et al (42) conducted a study in mice with murine AIDS. As is reported for aging, AIDS is accompanied by a cytokine dysregulation characterized by increased Th2 cytokine concentrations. Wang et al found that a 15-fold increase in dietary vitamin E (which is extremely high) partly restored the concanavalin A–stimulated production of IL-2 and IFN-γ by splenocytes and normalized the elevated production of IL-4, IL-5, and IL-6. Recently, Beharka et al (16) showed that in vitro addition of vitamin E to cultures of macrophages of normal old mice improved T cell IL-2 but not IL-4 production. In a study conducted in the elderly in which the influence of zinc on IFN-γ and IL-10 was investigated, in vitro zinc supplementation failed to reconstitute Th1 cytokine production (9).

From a public health point of view, it is important to consider the clinical relevance of the immunity measures used in this study. Several studies reviewed by Goodwin (43) as well as a study by Ferguson et al (44) showed a relation between decreased immune function and increased morbidity or mortality in patient populations as well as in healthy elderly populations. More specific for DTH, Roberts-Thomson et al (45) and Wayne et al (38) found a relation between anergy and mortality in elderly people. A change in DTH may be even more important than DTH per se as a predictor of clinical response; however, there are no data to support this theory (41). It has also been suggested that a dysregulation between Th1 and Th2 cytokines may be responsible for insufficient immune response in the elderly (9).

We found in a group of healthy elderly subjects that DTH increased more after 6 mo supplementation with 100 mg vitamin E than after placebo and we also found that this difference was even larger—although not signifcantly so after adjustment for multiple comparisons—in elderly subjects who had a low baseline DTH score or were less physically active. Therefore, we conclude that supplementation with 100 mg vitamin E may have a beneficial effect on cellular immune function and that this effect may be more proncounced in particular subgroups of the elderly. However, before vitamin E supplement use is widely proposed, new clinical trials should be conducted that show that vitamin E, possibly via an improvement of cellular immune responsiveness, is effective in reducing the morbidity of infectious diseases.

We thank the elderly volunteers for their wonderful cooperation in this study.

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