

Effect of 3 months vitamin E supplementation on indices of the cellular and humoral immune response in elderly subjects

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It has been suggested that decreased immune responsiveness in the elderly may be counteracted by the antioxidant vitamin E. In a 3-month double-blind placebo-controlled intervention trial among elderly subjects aged 65 years and over we studied the effects of a daily dose of 100 mg dl- α -tocopheryl acetate on the cellular immune responsiveness (n 52) measured by the *in vitro* response of peripheral blood mononuclear cells (PBMC) to the mitogens concanavalin A (ConA) and phytohaemagglutinin (PHA). Also effects on the humoral immune responsiveness (n 74) were investigated by measuring immunoglobulin (Ig)G, IgG4 and IgA antibody concentrations against various common antigens. In the vitamin E group plasma α -tocopherol increased by 51% ($P = 0.0001$) during intervention whereas no significant changes were observed in the control group. Initial proliferative PBMC responses differed between the vitamin E group and the control group whereas all other baseline characteristics were comparable. No significant changes were observed in cellular immune responsiveness when adjusted for initial values in either the control group or the vitamin E group and, after the trial period, responses in the two groups were not significantly different. Similarly, in the vitamin E group no significant changes were found in levels of IgG and IgA raised against *Penicillium* or IgG4 raised against egg, milk, or wheat proteins. In the control group small but significant increases in IgG anti-*Penicillium* ($P < 0.05$) and decreases in IgG4 against milk proteins ($P < 0.05$) were observed. Thus, the results of this study performed with the relatively low dose of 100 mg dl- α -tocopheryl acetate do not support the claims of a beneficial effect of vitamin E intake on the overall immune responsiveness of elderly subjects.

Vitamin E: Cellular immune response: Humoral immune response: Elderly

A decreased functioning of specific immune defence mechanisms with ageing (Weksler & Hutteroth, 1974; Kishimoto *et al.* 1980; Burns *et al.* 1990; Wayne *et al.* 1990; Makinodan, 1995) is presumed to contribute to increased morbidity and mortality in the elderly (Roberts-Thomson *et al.* 1974; Gardner, 1980; Murasko *et al.* 1988; Wayne *et al.* 1990; Goodwin, 1995). In addition to chronic diseases that accompany ageing that may lead to a decrease in immune responsiveness in the elderly, the ageing process itself is thought to play a major role too (Goodwin *et al.* 1982), highlighting the potential relevance of improving the immune response in 'healthy' elderly people. Increased lipid peroxidation and production of eicosanoids such as prostaglandin E₂ (PGE₂) may be important determinants in age-associated dysregulation of immune responsiveness. Vitamin E may improve immune responsiveness by counteracting these effects (Corwin & Shloss, 1980a; Goetzel, 1980; Likoff *et al.* 1981; Meydani *et al.* 1986, 1990, 1995; Hayek *et al.* 1994).

Effects of vitamin E supplementation on the cellular and humoral immune response in experimental animals have been reported extensively (Tengerdy *et al.* 1973, 1984; Corwin & Shloss, 1980*b*; Corwin *et al.* 1981; Larsen & Tollersrud, 1981; Bendich *et al.* 1986; Meydani *et al.* 1986; Reddy *et al.* 1987). Observational studies in healthy elderly human subjects found no positive associations between vitamin E intake or status and indicators of cell-mediated immune response (Goodwin & Garry, 1983; Chavance *et al.* 1989; Payette *et al.* 1990). However, these observational studies were cross-sectional and not designed to provide information on the effect of (long term) specific vitamin supplementation. In an intervention study among free-living healthy human elderly subjects with a daily dose of 800 mg dl- α -tocopheryl acetate for 30 d, Meydani *et al.* (1990) observed an overall improvement in cell-mediated immune response as shown by increased frequency and size of positive delayed type hypersensitivity skintest responses, interleukin-2 production and *in vitro* proliferative response of peripheral blood mononuclear cells (PBMC) to the mitogen concanavalin A (ConA). Also a reduced PGE₂ synthesis by PBMC was seen, which argues in favour of reduction of PGE₂ production as a possible working mechanism. In the same study no significant differences were found in humoral immune response measured as total serum concentrations of immunoglobulin (Ig)M, IgG and IgA, nor in the T-cell independent proliferative response of B cells to the mitogen *Staphylococcus aureus* Cowan. In another intervention study among institutionalized healthy elderly women, supplementation with 200 mg vitamin E for 1 year had no effect on total IgM, IgG and IgA antibody concentrations (Ziemiński *et al.* 1986).

The optimal dose for potential beneficial effects of supplemental vitamin E on immune variables in the elderly has not yet been determined. Studies investigating low or relatively (compared with other trials) low doses of supplemental vitamin E can contribute to the debate on whether it is warranted to advise elderly people to increase their intake of vitamin E-rich foods, or to explore the possibilities of enrichment of eligible food products or to advise vitamin E supplement use. But, at this point there is only limited evidence that the positive effects of vitamin E on immune responses, as found in animal experiments, generalize to human elderly subjects. Therefore, we have investigated the effects of a 3-month period of 100 mg α -tocopheryl acetate supplementation on cell-mediated immune responsiveness, expressed as *in vitro* response of PBMC to the mitogens ConA and phytohaemagglutinin (PHA), and on humoral immune responsiveness, assessed by measuring IgG, IgG4 and IgA antibody concentrations against various common antigens. To avoid inter-assay variation, inherent to the mitogen proliferation assays, isolated PBMC were stored in liquid N₂ and mitogen proliferative response of pre- and post-intervention PBMC of each individual were measured simultaneously in the same run.

METHODS

Population and design

The study was designed as a 3-month randomized double-blind placebo-controlled trial to investigate the association between vitamin E and health characteristics in elderly subjects, such as immune response, lung function and, as the main question, the effect on oxidation of LDL-cholesterol, and was conducted in 1994. In two consecutive steps a total of eighty-three apparently healthy elderly subjects, aged 67–85 years, were selected from a database of a population of 1012 elderly people in the city of Arnhem, The Netherlands (Van den Homberg, 1995). The first selection step included the following criteria: still living in Arnhem, not institutionalized, no use of prescribed drugs for cardiovascular disease (CVD), high blood pressure or diabetes mellitus; no reported cancer or chronic diseases of

the bowel, or stomach, kidney, or liver. In the second step, the 355 remaining persons were invited by mail and received a questionnaire on health status, smoking habits, and use of supplements and medication. Seventy-five subjects did not respond, 129 refused and 151 were willing to participate. From this latter group sixty-eight were not eligible because of the following criteria: use of vitamin supplements (A, A + D, C, E or multivitamin; (n 25), stopped smoking less than 5 years before (n 5), current treatment for CVD, heart attack or stroke (n 31), use of medication for hypertension or high cholesterol (n 6), or diabetes (n 2). The eighty-three eligible subjects were randomly assigned to intervention (n 42) and placebo groups (n 41). To ensure group comparability, subjects were prestratified before randomization on the following variables: smoking habits (current *v.* non-smokers), 5-year age groups and sex. The subjects received either vitamin E (100 mg dl- α -tocopheryl acetate; Hoffman-La Roche, Mijdrecht, The Netherlands; 50 mg twice daily) or placebo (lactose) capsules. Of the eighty-three subjects entering the study one subject in the vitamin E group who took only 15% of the distributed capsules was excluded from the data analysis. Eight subjects had missing values on baseline or post-intervention humoral immune response measurements. A random group of thirty subjects had missing values for baseline and/or post-intervention values for the mitogenic proliferation responses due to shortage of plasma or isolated blood mononuclear cells to perform all the tests. Data analysis was performed on complete data sets. Therefore, baseline and humoral immune characteristics are presented for seventy-four subjects (thirty-six in the placebo group and thirty-eight in the vitamin E group). For cell-mediated immune response, data on fifty-two subjects (twenty-five placebo and twenty-seven vitamin E group) are presented.

Data collection

At the beginning and end of the 3-month intervention period subjects were picked up at home and brought to the examination room at the University. Fasting blood samples were taken into EDTA for determination of antioxidant vitamin levels and for measurement of indices of the cellular and humoral immune response. Body weight, height and blood pressure were determined on both occasions as well. At baseline the subjects filled in a questionnaire on socio-demographic characteristics, physical activity and perceived health score. A semi-quantitative food-frequency questionnaire was administered and used to estimate antioxidant vitamin intake (Ocké *et al.* 1997). Subjects were asked to monitor intercurrent illness and/or medicine use in a diary. To improve and monitor compliance subjects were visited at home once during the trial. At the end of the study compliance was assessed by pill counts.

The study was approved by the Medical Ethical Committee of the Agricultural University and all subjects gave written informed consent to participate.

Laboratory measurements

Plasma was prepared under N₂ within 3 h after venepuncture and stored at -80° until analysis, to minimize analytical variation. Laboratory baseline and post-intervention measurements for each individual were performed in the same run. Concentrations of α -tocopherol, $\beta + \gamma$ -tocopherol, and β -carotene were measured in plasma by reverse-phase HPLC (adapted from Hess *et al.* 1991). The HPLC analyses were performed using a system from Thermo Separation Products (Freemont, CA, USA). The column was a prepacked 0.25 m \times 4.6 mm Vydac 201TP54, C₁₈ 300 Å (Hesperia, CA, USA). The mobile phase used for the Vydac column was methanol-tetrahydrofuran-water in the following proportions:

0 min, 89:2:9; 10 min, 98:2:0; 20 min 97:3:0; 30 min 90:10:0; 40 min, 90:10:0 by volume, and the flow rate was 1 ml/min. Detection after separation was carried out using two u.v. detectors, one for determination of carotenoids (u.v. 2000) and one (u.v.1000) for determination of the tocopherols.

Humoral immune response: antigen-specific immunoglobulins G and A. The following specific antibodies were measured by enzyme immunoassay essentially as described previously (Houba *et al.* 1996; Zock *et al.* 1996): IgG and IgA against a mixture of extracts of four different strains of *Penicillium* (Allergologisk Laboratorium Kopenhagen, Benelux, Houten, The Netherlands; art. no. 25-00) and IgG4 against egg (Laboratorium Diephuis, Groningen, The Netherlands; art. no. 58-04), milk (art. no. 58-01) and wheat (Houba *et al.* 1996) proteins. High-capacity microtitre plates (Greiner no. 655061; Greiner, Nuertingen, Germany) were coated overnight at 4° with the antigen preparations in phosphate-buffered saline (PBS). After blocking of free binding sites with gelatin-containing PBS-Tween (PBTG), duplicate 0.1 ml portions of sera diluted 1:100 (IgG4) and 1:500 (IgA, IgG) in PBTG were incubated in wells coated with the various antigens and in non-coated control wells. Binding of IgG, IgG4 or IgA was measured with peroxidase-labelled mouse monoclonal anti-human IgG (Central Laboratory of the Red Cross Blood transfusion Service, Amsterdam, The Netherlands (CLB) no. M1304), anti-human IgG4 (CLB no. M1331), or anti-IgA (CLB, no. M1354) respectively, followed by 30 min incubation with *o*-phenylenediamine (OPD). The reaction was read at 405 nm, terminated by addition of HCl, and then read at 492 nm. Absorbance values at 492 nm (A_{492}) > 3.0 were obtained by extrapolation from the A_{405} values according to the method of Doekes *et al.* (1993).

Each plate included a positive control (pooled plasma) and no-plasma controls in each type of coated and non-coated well. Antibody concentrations were expressed as A_{492}^* values corrected for values observed in control wells, as follows:

$$A_{492}^*(i, a_1) = [A_{492}(i, a_1) - A_{492}(0, a_1)] - [A_{492}(i, 0) - A_{492}(0, 0)]$$

in which $A_{492}(i, a_1)$ is the value obtained with plasma *i* on antigen a_1 , $A_{492}(0, a_1)$ the value obtained with buffer on antigen a_1 , and $A_{492}(i, 0)$ and $A_{492}(0, 0)$ are the analogous values obtained in non-coated wells.

Pre- and post-intervention plasma samples of each subject were tested in the same microtitre plate, to avoid inter-assay variation.

Cellular immune response: isolation and mitogenic stimulation of peripheral blood mononuclear cells. Within 6 h after venepuncture, PBMC were isolated from EDTA-blood by Ficoll-Hypaque (Pharmacia BiotechAB, Uppsala, Sweden) density gradient centrifugation (Boyum, 1968). PBMC were harvested and washed twice with sterile PBS and resuspended in 1 ml culture medium (RPMI 1640; Sigma-Aldrich Co. Ltd, Irvine, Strathclyde) with 100 ml/l dimethylsulfoxide (DMSO) (hybri-max, Sigma-Aldrich Co. Ltd) in polypropylene vials. The cells were frozen overnight at -80° and stored in liquid N₂ (-196°) until analysis.

For mitogenic stimulation, vials containing pre- and post-intervention PBMC were removed from the liquid N₂ simultaneously and thawed quickly at 37° in a 5% CO₂ incubator. DMSO was removed by washing with RPMI-1640. This washing procedure was repeated twice to ensure all DMSO had been removed, after which the pellets were resuspended in 750 µl RPMI-1640 and the concentration of viable cells was determined with the Trypan Blue exclusion method.

Portions of 0.5×10^6 cells were cultured in twenty-four-well plates (Costar, Cambridge, MA, USA) in 0.50 ml RPMI-1640 containing 2 mM-L-glutamine (Sigma),

50 μM 2-mercaptoethanol (Sigma), 30 $\mu\text{g/ml}$ gentamycin (Sigma), and 100 ml/l normal human serum, in the presence of PHA (Sigma) or ConA (Sigma), both at 1 and 5 $\mu\text{g/ml}$. Normal human serum was a pool of sera from five healthy department members; these sera had all been tested individually in pilot experiments, and were free of detectable mitogenic activity. They were heat inactivated by incubation at 56° for exactly 30 min followed by 15 min centrifugation at 1500 rev./min before storage. On each culture plate control wells were included with pre- and post-intervention PBMC of the same subject in the same medium without mitogens.

After incubation for 90 h at 37°, 5% CO₂ and 95–98% relative humidity, cells were washed twice in cold serum-free saline, and resuspended in the original volume (0.5 ml) of RPMI-1640, with glutamine, gentamycin and mercaptoethanol, but without human serum. In these suspensions metabolic cell activity was assessed by the MTT method (Mosmann, 1983) with adaptations taken from Denizot & Lang (1986). Thus, of each 0.5 ml suspension three 0.12 ml portions were applied to a ninety-six-well flat-bottom plate, mixed with 10 μl (10 mg/l) solution of 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) stock solution. After 4 h incubation 120 μl isopropanol was added to each well to stop MTT conversion and to dissolve the produced formazan, which was quantified spectrophotometrically at 570 nm.

Mitogenic stimulation was expressed as the difference in A₅₇₀ between the mean of duplicate cells cultured with mitogens and the control cell cultures.

Data analysis

Baseline results are expressed as means and standard deviations (SD) or median and 25–75% range. Mean baseline values and mean individual changes during intervention were compared between the study groups by using the unpaired *t* test in the case of normal distribution and the Mann–Whitney test. Similarly, mean individual changes over the intervention period for the control and vitamin E groups were tested with the paired *t* test or with the Wilcoxon-signed rank test for paired comparisons within groups.

Multiple linear regression was used to adjust differences in change in proliferative response values between the placebo group and the vitamin E group for initial values (Chambless & Roebuck, 1993). Presented correlations are expressed as Spearman correlation coefficients. All analyses were performed using the statistical package SAS (Statistical Analysis Systems, 1989).

RESULTS

Table 1 presents the mean baseline characteristics of the placebo and vitamin E-supplemented groups. Pre-stratification on sex, smoking behaviour and 5-year age groups was successful, as shown by equal distribution of these variables in both study groups. Subjects participated for, on average, 3 months in the study (84 d; Table 1) with a variation from 61 to 94 d. Differences in supplementation period, mainly due to measuring during the holiday period (May–June), were not associated with immune response nor with particular population characteristics. The number of subjects not reporting any intercurrent illness or medicine use during the trial was slightly higher in the vitamin E group (*n* 21) than in the control group (*n* 16).

Baseline plasma antioxidant vitamin concentrations and estimated dietary antioxidant vitamin intake did not differ significantly between intervention and control groups (Table 2). In the vitamin E group plasma α -tocopherol increased during intervention by 51% (mean

Table 1. Characteristics of elderly subjects participating in a 3-month intervention trial with vitamin E

(Mean values with their standard deviations or frequency (%))

	Control group (n 36)		Vitamin E group (n 38)	
	Mean	SD	Mean	SD
Age (years)	74.5	5.2	74.2	5.2
Male (%)	67		61	
Smokers (%)	25		24	
Intervention period (d)	83.7	6.8	84.2	4.5
Pills taken (%)	93.9	9.0	93.0	6.9
Health score*	8.2	1.3	8.2	1.3
Height (m)	1.70	0.09	1.70	0.09
BMI (kg/m ²)	25.1	3.2	25.9	3.2
No reported illness or medicine use †(%)	44		55	
Reported illness (d) ‡	5.5	8.7	3.5	3.4

* Rating on a scale from 1 (worst) to 10 (best).

† Based on self-reported occurrence of illness and use of medicine, recorded in a health diary during the 3-month intervention period.

‡ Only for the group who reported illness or medicine use in their diary: placebo group, n 20, and vitamin E group, n 17.

16.7 (SD 12.7) $\mu\text{mol/l}$, $P = 0.0001$). In the vitamin E group the increase in α -tocopherol was correlated with an observed decrease of plasma $\beta + \gamma$ -tocopherol (Spearman correlation $r = 0.5$, $P = 0.006$). At baseline only one subject had a plasma α -tocopherol level below the cut-off value of 11.6 $\mu\text{mol/l}$ for risk of vitamin E deficiency in human subjects and none below the 2.2 μmol α -tocopherol/mmol cholesterol value. Taking the optimal levels to prevent chronic disease as postulated by Gey (1994), 32 % fell below the 30 μmol plasma α -tocopherol/l limit and 18 % below the 5.2 μmol α -tocopherol/mmol cholesterol value. In this study similar values for the reported immune variables were observed between subjects above and below the optimal α -tocopherol levels mentioned earlier.

Humoral immune response; immunoglobulin G and A antibody concentrations

At baseline, median concentrations of IgA and IgG raised against *Penicillium* and IgG4 raised against three different food antigens were comparable in vitamin E and placebo groups (Table 3). In the vitamin E-supplemented group no significant changes in antibody concentrations were observed after 3 months. In the control group a small but significant increase was seen for the IgG concentration against *Penicillium* (median change A_{492} 0.1; $P = 0.01$) and a small decrease in IgG4 against milk protein (median change $A_{492} - 0.01$; $P = 0.04$). During the intervention period, similar changes were observed in the intervention and placebo groups. Baseline and 3-month antibody concentrations were highly correlated (r 0.9 for all antibody concentrations in both groups). Baseline concentrations of IgG4 against the three different food antigens showed strong mutual correlations ($0.6 < r < 0.7$; $P = 0.0001$).

Baseline antibody concentrations for the total study population were not significantly correlated with age, BMI, antioxidant plasma concentrations or antioxidant vitamin intake. Higher baseline values of IgG4 against food antigens were seen for females than for males (IgG4 against milk protein: median A_{492} 2.6 (25–75 % interval 0.3–4.3) for females and 0.5 (interval 0.03–2.2) for males, $P = 0.03$). These sex differences persisted when comparing

Table 2. Plasma antioxidant vitamin concentrations and antioxidant vitamin intakes of elderly subjects during a 3-month intervention trial with vitamin E†

	Control group (n 36)				Vitamin E group (n 38)			
	Baseline		Change		Baseline		Change	
	Mean or median	SD or 25-75 %	Mean or median	SD or 25-75 %	Mean or median	SD or 25-75 %	Mean or median	SD or 25-75 %
Plasma antioxidant vitamins (µmol/l)								
α-Tocopherol	34.4	7.1	-0.8	3.7	33.0	8.1	16.7**	12.7
β + γ-Tocopherol	2.7	2.1-4.3	-0.06	-0.4-0.4	3.2	2.1-4.2	-1.2**	-1.9-0.6
β-Carotene	0.3	0.3-0.5	0.01	-0.06-0.03	0.3	0.2-0.4	0.03	-0.02-0.09
Antioxidant vitamins intake (mg/d)								
Energy (MJ/d)	7.6	2.1			7.6	1.7		
Vitamin E	9.2	7.2-15.8			11.0	8.2-13.4		
β-Carotene	1.3	0.9-1.5			1.4	1.0-1.6		
Vitamin C	100.1	73.7-143.2			84.0	73.2-130.9		

** Mean or median values were significantly different from those for the control group, $P < 0.01$.

† For details of subjects and procedures, see Table 1 and pp. 762-764.

Table 3. Concentrations of antibodies against common antigens (absorbance at 492 nm) in elderly subjects and changes to these concentrations during a 3-month intervention trial with vitamin E†

(Median values and 25–75 % range)

	Control group (n 36)				Vitamin E group (n 38)			
	Baseline		Change		Baseline		Change	
	Median	25–75 %	Median	25–75 %	Median	25–75 %	Median	25–75 %
IgA, <i>Penicillium</i>	1.2	0.5–2.0	0.01	–0.1–0.1	1.0	0.6–2.2	–0.01	–0.1–0.1
IgG, <i>Penicillium</i>	2.8	1.5–3.6	0.1*	–0.0–0.3	3.0	2.4–4.0	–0.04	–0.2–0.2
IgG4, egg protein	3.3	2.1–5.5	–0.1	–0.3–0.3	3.9	0.6–5.8	0.02	–0.1–0.2
IgG4, milk protein	1.1	0.1–3.1	–0.01*	–0.2–0.0	1.0	0.0–4.2	0.0	–0.1–0.1
IgG4, wheat protein	0.2	0.0–2.6	0.0	–0.1–0.0	0.5	0.1–2.4	0.0	–0.1–0.1

Ig, immunoglobulin.

* Changes were significantly different from zero, $P < 0.05$.

† For details of subjects and procedures, see Table 1 and pp. 762–765.

non-smoking males (n 30) and females (n 26). No sex differences for IgG and IgA raised against *Penicillium* were observed. The difference between smokers and non-smokers was evaluated among men because only one female smoker was present. Only for IgG against *Penicillium* was a significantly ($P = 0.0004$) lower baseline value for smokers (median 2.1 (interval 1.2–2.7)) seen as compared with non-smokers (median 3.5 (interval 2.7–4.3)).

Cellular immune response; mitogen stimulation of peripheral blood mononuclear cells with phytohaemagglutinin and concanavalin A

Data for cellular immune responses were available for a subpopulation of fifty-two subjects from the total study population. Baseline characteristics of this subpopulation were similar to those given for the total study population in Tables 1 and 2.

At baseline lymphocyte proliferation to both mitogens ConA and PHA (5 $\mu\text{g}/\text{ml}$) was significantly lower ($P = 0.04$) in the control group (Table 4). Furthermore, changes in PHA proliferative responses during intervention were negatively correlated with initial values ($r = -0.5$, $P < 0.02$ for both study groups). To obtain an unbiased estimate, differences in change in proliferative response between the vitamin E group and the placebo group were adjusted for initial proliferative response values. As shown in Table 4 the adjusted treatment effect indicates that vitamin E had no effect on mitogenic stimulation of PBMC with PHA and ConA. To explore a potential effect of baseline α -tocopherol plasma values on immune response we analysed separately the subjects in the lower two tertiles of baseline α -tocopherol values. This left thirty-two subjects (n 16 for both control and vitamin E groups) for analysis, with mean α -tocopherol baseline values of 28.9 (SD 4.9) $\mu\text{mol}/\text{l}$ and 27.5 (SD 5.6) $\mu\text{mol}/\text{l}$ and increases during intervention of 0.4 (SD 3.4) and 11.2 (SD 9.6) $\mu\text{mol}/\text{l}$ in the control and vitamin E groups respectively. The results on mitogenic response for this sub-sample did not differ from those obtained from the total sample.

A_{570} values for unstimulated PBMC were similar for control and vitamin E group showing means of 0.10 (SD 0.05) and 0.11 (SD 0.08) respectively. Correlations between baseline and post-intervention measurements for PHA-stimulated PBMC were r 0.5 ($P = 0.006$) and r 0.8 ($P = 0.0001$) for control and vitamin E groups respectively. For ConA, correlations of r 0.6 ($P = 0.0008$) and r 0.7 ($P = 0.0001$) were observed (see Fig. 1).

Table 4. Mitogenic stimulation of peripheral blood mononuclear cells from elderly subjects, and changes in these characteristics during a 3-month intervention trial with vitamin E†
(Mean values and standard deviations)

Stimulant	Control group (n 25)				Vitamin E group (n 27)				Treatment effect § Change (vitamin E - control group)			
	Baseline		Change		Baseline		Change		Unadjusted		Adjusted for initial values	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SE	Mean	SE
PHA, 5 µg/ml	0.41	0.23	0.15	0.21	0.67*	0.32	0.03*	0.20	-0.12†	0.06	-0.04	0.06
ConA, 5 µg/ml	0.26	0.09	0.13	0.17	0.40*	0.20	0.06	0.17	-0.07	0.05	-0.05	0.05

PHA, phytohaemagglutinin; ConA, concanavalin A.

* Mean values were significantly different from control group, $P < 0.05$.

† Mean changes were significantly different from zero, $P < 0.05$.

‡ Proliferation was assessed by the MTT method and expressed as the absorbance value (at 570 nm) of the formazan produced. For details of subjects and procedures, see Table 1 and pp. 762-765.

§ Treatment effect is expressed as difference in 3-month changes in mitogenic stimulations between vitamin E and placebo-supplemented groups and expressed as the β_1 and standard error from the regression equation: $X_2 = \beta_0 + \beta_1 T + \beta_2 X_1$, where X_2 is change in proliferative mitogenic response, X_1 is baseline proliferative value and T is treatment group (1 = intervention group, 0 = placebo group).

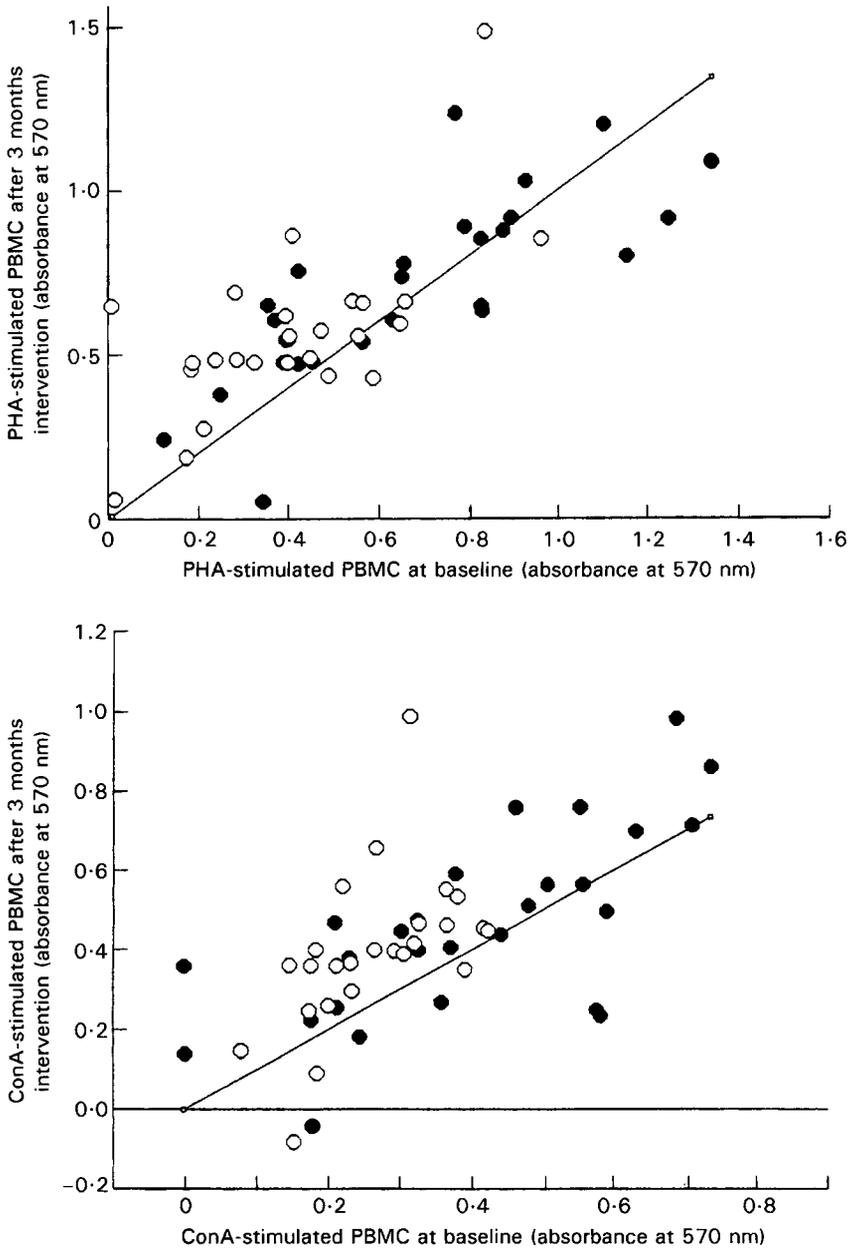


Fig. 1. Stimulation of peripheral blood mononuclear cells (PBMC) from elderly subjects with the mitogens phytohaemagglutinin (PHA) and concanavalin A (ConA) measured at baseline *v.* measurements made after 3 months intervention with vitamin E (●) or a placebo (○). For Spearman correlation coefficients, see pp. 768–771. Proliferation was assessed by the MTT method and expressed as the absorbance at 570 nm of the formazan produced. The line shown is the plot $X = Y$.

Baseline values for proliferative response were not significantly correlated with age, BMI, antioxidant plasma concentrations or antioxidant vitamin intake. No differences were found between men and women or between smokers and non-smokers in baseline values.

Similar results were observed for proliferative responses with 1 μ g mitogen/l (results not shown).

DISCUSSION

In this 3-month intervention trial among apparently healthy elderly subjects no effect was observed of 100 mg α -tocopheryl acetate on the *in vitro* proliferative response of PBMC to the mitogens PHA and ConA. Also antibody concentrations against various common antigens were not affected by vitamin E supplementation.

Ageing itself, and diseases that frequently accompany ageing, are thought to be factors explaining the decline in immune responsiveness with ageing. Goodwin *et al.* (1982) found no difference in cellular immune responsiveness between healthy and chronically ill elderly subjects. This suggests that the major determinant of decreased cellular immune function is age *per se* and not age-associated diseases. Therefore improving immune responsiveness in healthy elderly people may be of great public health importance. The present study population was selected as 'healthy' using a questionnaire on disease and medicine use, and therefore cannot be considered representative of the elderly population in general. Underlying subclinical diseases may well have been present but are not expected to have influenced the comparison between the two study groups because of randomization. Comparison within study groups of those not reporting illness during intervention with those reporting illness, did not reveal any differences in studied immune variables.

A great variability inherent to the mitogen proliferation assays has been mentioned by Meydani *et al.* (1990). To avoid inter-assay variation, isolated PBMC were stored in liquid N₂ and mitogen proliferative responses of pre- and post-intervention PBMC for each individual were measured simultaneously in the same run. The storage period of PBMC was not significantly associated with the proliferative responses.

Intra-individual variation in the mitogenic response measured at different time points may lead to regression towards the mean and could have affected our results, as we observed unexpected differences in initial values in mitogenic response between vitamin E and control groups. Baseline PHA and ConA responses in the control group were significantly lower than in the vitamin E group, while post-intervention responses were similar in both groups and at the level of the vitamin E group at baseline. Therefore we adjusted the intervention effect (changes in proliferative response between vitamin E and control group) for initial values by means of multiple linear regression (Chambless & Roebuck, 1993).

Vitamin E is thought to exert its effect on T-cell response and interleukin-2 production. Therefore an effect on humoral immune response would be primarily expected for T-cell-dependent antibody responses. Ziemiński *et al.* (1986) and Meydani *et al.* (1990) did not show an effect of vitamin E on total antibody (IgG, IgM, IgA, IgD) concentrations. Total IgG serum concentrations are, however, a rather crude estimate of humoral immune responsiveness. Therefore, we decided to measure specific antibodies to various common antigens like *Penicillium*, milk, wheat and egg proteins to which most individuals are probably constantly exposed, and against which specific IgG and IgA antibodies are produced.

Taking into account the reported steady state of plasma α -tocopherol after 4–5 d following chronic administration of vitamin E (Dimitrov *et al.* 1991), the lifetime of activated B-cells (4 weeks) and the half-life of antibodies in circulation (3 weeks), the intervention period of 3 months seems adequate for detecting important changes in antibody production.

Improvement of indices of the cellular and humoral immune response by vitamin E has been extensively demonstrated in animal experiments. Reviews on the subject (e.g. Meydani *et al.* 1995) indicate important implications for the human situation, although this is supported by only a few studies in human populations. The first intervention study among elderly people on the effect of vitamin E on a large number of cellular and humoral immune indices was reported by Meydani *et al.* (1990). They showed increases in the proliferative response of PBMC to ConA but not to PHA after daily supplementation with 800 mg α -tocopheryl acetate for 30 d. The rise in plasma α -tocopherol in their study was 3-fold while in our study with 100 mg α -tocopheryl acetate the relative increase was 51%. The smaller increase in α -tocopherol in blood and probably also in PBMC (not measured in the present study but in the study by Meydani *et al.* (1990) also 3-fold) might not have been enough to improve the *in vitro* mitogen proliferation of PBMC. This could mean that very high vitamin E doses are needed to improve mitogen-stimulated proliferation of PBMC. This is supported by another intervention in elderly subjects reported by Meydani *et al.* (1994), with administration of different vitamin E doses (60, 200 and 800 mg vitamin E for 120 d), and in which a significant increase in mitogenic response to ConA was observed for the 800 mg dose only. An increased response to delayed-type hypersensitivity tests was, however, seen in all supplemented groups.

Alternatively, our results may be due partly to the relatively high baseline plasma α -tocopherol values in the present study as compared with, for example, the trial of Meydani *et al.* (1990). Chandra (1992) found, in a 1 year multivitamin supplementation (including 44 mg vitamin E) trial among healthy elderly subjects, that improvement in immunological responses (increase in natural killer cells, lymphocyte proliferation to PHA, a higher antibody response to influenza vaccine, and a lower frequency of infection-related illness) was greater among subjects who at baseline had low plasma nutrient values. Therefore we tested the intervention effect for the subjects in the lower two tertiles of the baseline plasma values revealing similar mean plasma α -tocopherol values to those in the study of Meydani *et al.* (1990) but still found no effect on mitogenic response between the control and the vitamin E group. Therefore, it does not seem likely that relatively high baseline vitamin E levels are to be held responsible for not finding an effect of vitamin E supplementation on cellular immune response in the present study.

Our study in the elderly did not show improvements in specific immune indices after supplementation with 100 mg α -tocopheryl acetate for 3 months. This does not mean that relatively low doses of vitamin E might not affect other immune variables in elderly subjects (see Meydani *et al.* 1994). Studies on different doses of vitamin E and different immune variables will improve the available information on the overall possible positive effects and working mechanism and should provide necessary background information on the optimal doses. Better insight into improvement of the immune responsiveness in elderly people by vitamin E might warrant studies relating vitamin E to morbidity and mortality, especially those factors contributing to infections.

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