Modulated mitogenic proliferative responsiveness of lymphocytes in whole-blood cultures after a low-carotene diet and mixed-carotenoid supplementation in women[1–3]

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ABSTRACT To determine the effects of dietary carotenes on the mitogenic proliferative responsiveness of blood lymphocytes in vitro, nine premenopausal women were fed a low-carotene diet for 120 d. Low-dose β-carotene (0.5 mg/d) was given to five subjects on days 1–60, while four received a placebo. All subjects received a low-dose β-carotene (0.5 mg/d) supplement on days 61–120, plus a carotenoid complex on days 101–120. The mean (± SEM) serum β-carotene concentration for the combined β-carotene supplemented and placebo subjects ($n = 9$) was not significantly reduced from that on day 1 (1.27 ± 0.24 μmol/L) on days 60 (0.66 ± 0.14 μmol/L) and 100 (0.91 ± 0.38 μmol/L), but on day 120 (3.39 ± 0.44 μmol/L) it was increased above that on days 1, 60, and 100. Maximum mitogenic proliferative responsiveness of blood lymphocytes in vitro to optimal dose phytohemagglutinin (PHA) was reduced on days 60 ($P = 0.025$) and 100 ($P < 0.0001$), but corrected itself on day 120 to a value above those on day 1 ($P = 0.04$), day 60 ($P = 0.0001$), and day 100 ($P < 0.0001$). Present findings show that a diet low in carotene had a suppressive effect on the maximum mitogenic proliferative responsiveness of blood lymphocytes in vitro, which was not corrected with low-dose β-carotene supplementation but was with a carotenoid complex from vegetables rich in carotenoids. *Am J Clin Nutr* 1997;65:871–5.

KEY WORDS β-Carotene, carotenoids, T lymphocyte proliferation, mitogenic responsiveness, whole-blood cultures, phytohemagglutinin

INTRODUCTION

The positive association between dietary intake of carotenoid-rich fruit and vegetables and decreased risk of specific cancers is consistently documented across observational epidemiologic research (1–9). The mechanism underlying this association is unclear. One possible mechanism is enhancement of immune responses by carotenoids (10, 11), noncarotenoid phytonutrients, or both (12, 13). Studies in animals show an immunoenhancing effect by isolated β-carotene (14, 15). Similar findings in humans have been inconsistent. Part of this may relate to the use of different cell culture systems for the measurement of the mitogenic proliferative responsiveness of blood lymphocytes in β-carotene supplementation studies. Using peripheral blood mononuclear cells (PBMCs), which contain plastic-adherent and nonadherent mononuclear leukocytes (herein referred to as adherent and nonadherent cells, respectively) cultured in autologous serum, van Poppel et al (16) found increased mitogenic proliferative responsiveness of blood lymphocytes in vitro to phytohemagglutin (PHA) but not to concanavalin A (ConA) in male smokers supplemented for 14 wk with 20 mg β-carotene/d. This enhancement was not observed when the PBMCs were cultured in medium containing heterologous, fetal bovine serum. Moriguchi et al (17) found increased mitogenic proliferative responsiveness of blood lymphocytes in vitro to PHA and ConA in young healthy male nonsmokers supplemented for 30 d with 30 mg β-carotene/d when the PBMCs were purified further to consist primarily of nonadherent cells and cultured as peripheral blood lymphocytes (PBLs) in heterologous serum. Using PBMCs, but cultured in nonautologous human serum type AB, Daudu et al (18) did not observe changes in mitogenic proliferative responsiveness of blood lymphocytes in vitro to PHA or ConA from healthy premenopausal women experiencing β-carotene depletion and repletion. Thus, a β-carotene modulating effect on mitogenic proliferative responsiveness of blood lymphocytes in vitro was found when the cells were cultured as PBMCs, which contain adherent and nonadherent cells, in the presence of autologous plasma (16) or when nonadherent cells, with a reduced number of adherent cells, were cultured as PBLs in heterologous serum (17). The modulating effect, however, was not found if the PBMCs were cultured in tissue culture medium containing heterologous serum (16) or nonautologous human serum type AB (18). To mimic, as much as possible, the in vivo state we chose to use the whole-blood culture method (19–22) to determine the effects of a low-carotene diet and supplementation with low-dose β-carotene (0.5 mg/d) and mixed carotenoids from vegetables on mitogenic proliferative responsiveness of blood lymphocytes in vitro to various doses of PHA.

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[2] Reference to a company or product name does not imply approval or recommendation of the product by the US Department of Agriculture to the exclusion of others that may be suitable.

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Received March 7, 1996.

Accepted for publication October 14, 1996.

SUBJECTS AND METHODS

Twelve healthy, normal-weight, premenopausal women aged 33.3 ± 7.5 y (range: 23–43 y) were selected for the study. One subject did not complete the study and two were on a different schedule from the main group, so their results were not included in the data analysis. All subjects lived in the metabolic research unit (MRU) of the US Department of Agriculture, Western Human Nutrition Research Center (WHNRC) at the Presidio of San Francisco, throughout the 120-d study. The study protocol was approved by the Human Subject Review Committee of the University of California, Davis, and the US Department of Agriculture Review Committee at Tufts University. It was conducted in accord with the Helsinki Declaration of 1975 as revised in 1983.

Research design

The subjects consumed a low-carotene diet made up of familiar, commercially available foods at proportional energy intakes of 55%, 14%, and 33% for carbohydrate, protein, and fat, respectively, throughout the 120-d study. The diet contained ≈0.075 mg β-carotene/d (estimated to be 90% of carotenoid intake), which is ≈4.1% of the estimated 1.8 mg/d intake by US women of reproductive age (23). On study days 1–60, five randomly selected subjects received 0.5 mg β-carotene/d from dry carotene beadlets (lot 14240; Hoffmann-La Roche Inc, Nutley, NJ) and the remaining four subjects received carotene-free beadlets (lot 312581; Hoffmann-La Roche Inc). All subjects received 0.5 mg β-carotene/d on study days 61–120, and during the final 20 d (days 101–120) of the study they received additional carotenoids from three capsules of mixed carotenoids per day (Neo-Life Company of America, Fremont, CA) (**Table 1**). All subjects were allowed to exercise at a fixed activity level that caused no major change in body weight, body composition, or resting metabolic rate. Body weights were kept constant (± 1 kg) by energy intake adjustments. The menu plan was a 6-d rotation of familiar foods low in carotene-rich fruit, vegetables, and juices. All subjects were given supplemental vitamins and minerals at ≈100% of the recommended dietary allowance (RDA) of all established micronutrients (25). Retinol and carotenes from the diets, dietary supplements, and serum samples were extracted and analyzed by using HPLC methods reported previously (26, 27).

Blood collection for whole-blood lymphocyte analysis

From each subject a 3-mL sample of fasting blood was collected from the antecubital vein into a sterile evacuated tube containing 45 U (US Pharmacopeia) sodium heparin (Becton Dickinson Co, Rutherford, NJ) for lymphocyte proliferation. The blood was shipped by air from the WHNRC in the Presidio of San Francisco to the Immunology Laboratory of the Beltsville Human Nutrition Research Center (BHNRC) in Beltsville, MD, by an overnight delivery service. The length of time between collection and processing of blood for lymphocyte proliferation in vitro was ≈26 h for each time period.

TABLE 1
Measured carotenoid concentration of a capsule containing a mixture of carotenoids[1,2]

Carotenoid	Capsule content	Supplement intake
	mg	*mg/d*
α-Carotene	0.466	1.398
β-Carotene	1.102	3.306
β-Cryptoxanthin	0.039	0.117
Lutein/zeaxanthin	0.497	1.491
Lycopene	0.221	0.663

[1] Carotenoid Complex; Neo-Life Company of America, Fremont, CA.
[2] Determined by HPLC (24).

Leukocyte numbers

Total leukocyte and partial differential counts were determined on fasting blood from the group collected in K_3EDTA-treated evacuated tubes on study days 1, 60, and 120 by using a Coulter JT Blood Analyzer (Coulter Corp, Miami) according to standard operational procedures. Values were not available from study day 100.

Mitogenic proliferative responsiveness

A slightly modified version of a previously described (28) whole-blood method for analysis of mitogenic proliferative responsiveness of blood lymphocytes in vitro to PHA was used in the present study. In brief, fasting blood collected in heparin was diluted 1:4 with RPMI-1640 tissue culture medium (Sigma Chemical Co, St Louis) in polystyrene tubes (Falcon; Becton Dickinson Co) for preparation of the whole-blood cultures. The RPMI-1640 contained L-glutamine (Sigma Chemical Co) at 2.0 mmol/L and penicillin-streptomycin at 100 000 U/L and 100 mg/L, respectively, referred to herein as RPMI-1640. The stock solution of PHA was prepared in RPMI-1640, sterilized by filtration, dispensed as aliquots, and stored at −20 °C. The cultures were prepared in triplicate with the following components added in order: *1*) 50 μL blood diluted 1:4, *2*) 50 μL RPMI-1640 alone (unstimulated) or with PHA at designated concentrations, and *3*) 100 μL RPMI-1640 per well of round-bottom 96-well tissue culture plate (Corning Glass Works, Corning, NY). The cultures contained a final volume of 200 μL, with the final blood dilution at 1:16.

Incorporation of [^{3}H]thymidine [specific activity of 248 GBq (6.7 Ci)/mmol); Dupont NEN Products, Boston] in DNA synthesis was used as an estimate of the proliferative activity of PHA-responsive blood lymphocytes in cultures without (background) and with PHA. Eight sets of triplicate whole-blood cultures received PHA (Sigma Chemical Co) at 0.0 (unstimulated), 0.125, 0.25, 0.5, 1, 2, 4, or 8 μg/culture, and were incubated for 72 h at 37 °C in an incubator with 5% CO_2 and 95% humidified air. Twenty hours before termination of incubation, 37 KBq (1.0 μCi) [^{3}H]thymidine was added to each culture. On completion of incubation the cell cultures were harvested (Skatron Inc, Sterling, VA) onto fiberglass filters. The [^{3}H]thymidine-labeled DNA on individual filtermat disks with scintillant (Ready Safe; Beckman, Palo Alto, Ca) were counted in a Beckman LS 3801 scintillation counter by using a single-label dpm program with the activity reported as dpm/culture. Maximum mitogenic proliferative responsiveness of blood lymphocytes in vitro to PHA was established by identification of the highest median value from the sets of triplicate cultures. Maximum activity was most frequently observed at PHA doses of 2.0 and 4.0 μg/whole-blood culture. The proliferative responsiveness is expressed as mean (+ SEM) radioactivity (in Bq).

Statistics

Each variable was analyzed by fitting a two-way mixed model. The fixed-effect variable was study days, and the random-effects variable was subject. In addition, a variance-covariance structure was modeled to account for the correlation between repeated measurements on a given subject. For each variable, the mean at each study day was compared with baseline by using Tukey's multiple-comparison procedure (29). The critical value was selected so that the probability of at least one false-positive result (type I error) was $\leq 5\%$.

Some variables were transformed before being analyzed (with a $\log_{10}$ or square root transformation) to correct for variance heterogeneity, distributional problems, and nonadditivity. Transforming the means back to the original scale gives an estimate of the median of the original population. The appropriate transformation was selected on the basis of the Box-Cox (30) power transformation method. The SAS computer software program (SAS Institute Inc, Cary, NC) was used for analyses.

On study day 60, the five subjects supplemented with 0.5 mg β-carotene/d and the four supplemented with placebo during days 1–60 showed no difference ($P > 0.05$) in blood lymphocyte proliferation. Because of this lack of difference, the two subgroups were combined to form a single group for all statistical analyses presented in this report.

RESULTS

Serum β-carotene

Mean ($\pm$ SEM) concentrations of serum β-carotene for the combined group ($n = 9$) were not significantly decreased from baseline (day 1; 1.27 $\pm$ 0.24 μmol/L) on study days 60 (0.66 $\pm$ 0.14 μmol/L) and 100 (0.91 $\pm$ 0.38 μmol/L). On study day 120 (3.39 $\pm$ 0.44 μmol/L) after 20 d of supplementation with three capsules of mixed carotenoids per day, the group showed a significantly ($P < 0.05$) higher mean concentration of serum β-carotene than on study days 1, 60, and 100. More results from this study on the concentrations of serum β-carotene and other carotenoids will be reported elsewhere.

Leukocyte numbers

The group did not show a significant ($P > 0.05$) change in absolute number of lymphocytes or midsize mononuclear leukocytes, predominately large lymphocytes and monocytes, throughout the study. On study days 1, 60, and 120, respectively, the mean absolute numbers of lymphocytes and midsize mononuclear leukocytes were 2.1, 1.9, and 2.0 $\times$ 10^9/L, and 0.4, 0.3, and 0.3 $\times$ 10^9/L, respectively.

Mitogenic proliferative responsiveness

Presented in **Figure 1** are the mean maximum and individual mitogenic proliferative responses of blood lymphocytes in whole-blood cultures to suboptimal (0.125, 0.25, 0.5, and 1.0 μg PHA), optimal (2.0 and 4.0 μg PHA), and postoptimal (8.0 μg PHA) concentrations (μg/culture) of PHA for maximum activity by the group on study days 1, 60, 100, and 120. In comparison with baseline (day 1), the group showed significantly reduced maximum and individual optimal dose (2.0 and 4.0 μg PHA) mitogenic proliferative responses of blood lymphocytes in vitro to PHA on study days 60 and 100. Supplementation of the group on study days 101–120 with three capsules of mixed carotenoids per day corrected the suppressed lymphocyte proliferative responses observed on study days 60 and 100, and significantly elevated the response on study day 120 over that on days 1, 60, and 100. Except for 0.25 μg PHA/culture on study day 100, mean mitogenic proliferative responsiveness of blood lymphocytes in vitro to suboptimal doses of PHA (0.125, 0.25, and 0.5 μg) for maximum activity failed to show differences in lymphocyte proliferation for the group throughout the study. At the immediate preoptimal dose (1.0 μg) of PHA for maximum mitogenic proliferative responsiveness of blood lymphocyte in vitro, the group showed a trend toward reduced lymphocyte proliferation on study day 100 and significantly increased activity on study day 120 over days 1 and 100. At the postoptimal dose (8.0 μg) of PHA for maximum mitogenic proliferative responsiveness of blood lymphocytes in vitro, the group showed significantly reduced lymphocyte proliferation on study days 60 and 100 in comparison with study days 1 and 120, but no longer showed a difference between study days 1 and 120.

Intersubject CVs for the mean mitogenic proliferative responses of blood lymphocytes in vitro to PHA were smallest for the maximum, optimal (2.0 and 4.0 μg/culture), and postoptimal (8.0 μg/culture) dose responses, intermediate for the immediate preoptimal (1.0 μg/culture) dose response, and largest for the suboptimal (0.125, 0.25, and 0.5 μg/culture) dose responses. Based on data that were $\log_{10}$ or square root transformed for normalization of data distribution, the mean CVs throughout the study for PHA doses of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 μg/culture, and the maximum responses were 13.9, 19.3, 11.7, 6.5, 3.5, 3.4, 3.3, and 3.0 μg/culture, respectively.

DISCUSSION

Using whole-blood cultures, which contain nonadherent and adherent lymphoid cells and autologous plasma, we found decreased maximum mitogenic proliferative responsiveness of human blood lymphocytes in vitro to optimal-dose PHA in adult women fed a diet low in carotenes, of which 90% were β-carotene (Figure 1). We found that the suppressed responsiveness was not corrected by supplementation of the subjects with low-dose β-carotene (0.5 mg/d) but was corrected by supplementation for 20 d with concentrates of mixed carotenoids. These findings do not agree with those of a comparatively similar study by Daudu et al (18) using density-gradient-prepared PBMCs, which also contain adherent and nonadherent lymphoid cells, cultured in nonautologous human serum type AB. Although there were many similarities between the two studies, there were two major technical differences that may have resulted in the lack of agreement. Results reported by Daudu et al (18) were on blood samples collected and processed on the same day at the WHNRC for lymphocyte proliferation in vitro. In the present study, the blood was collected at the WHNRC and shipped by air with an overnight air delivery service to the BHNRC for analysis of lymphocyte proliferation in whole-blood cultures. Thus, samples in the present study were processed $\approx$26 h after collection. It is unclear in the present study what effect overnight shipment and a 24-h delay in processing of blood samples for mitogenic proliferative responsiveness of lymphocytes in vitro had on the results. However, in experiments completed recently, we found

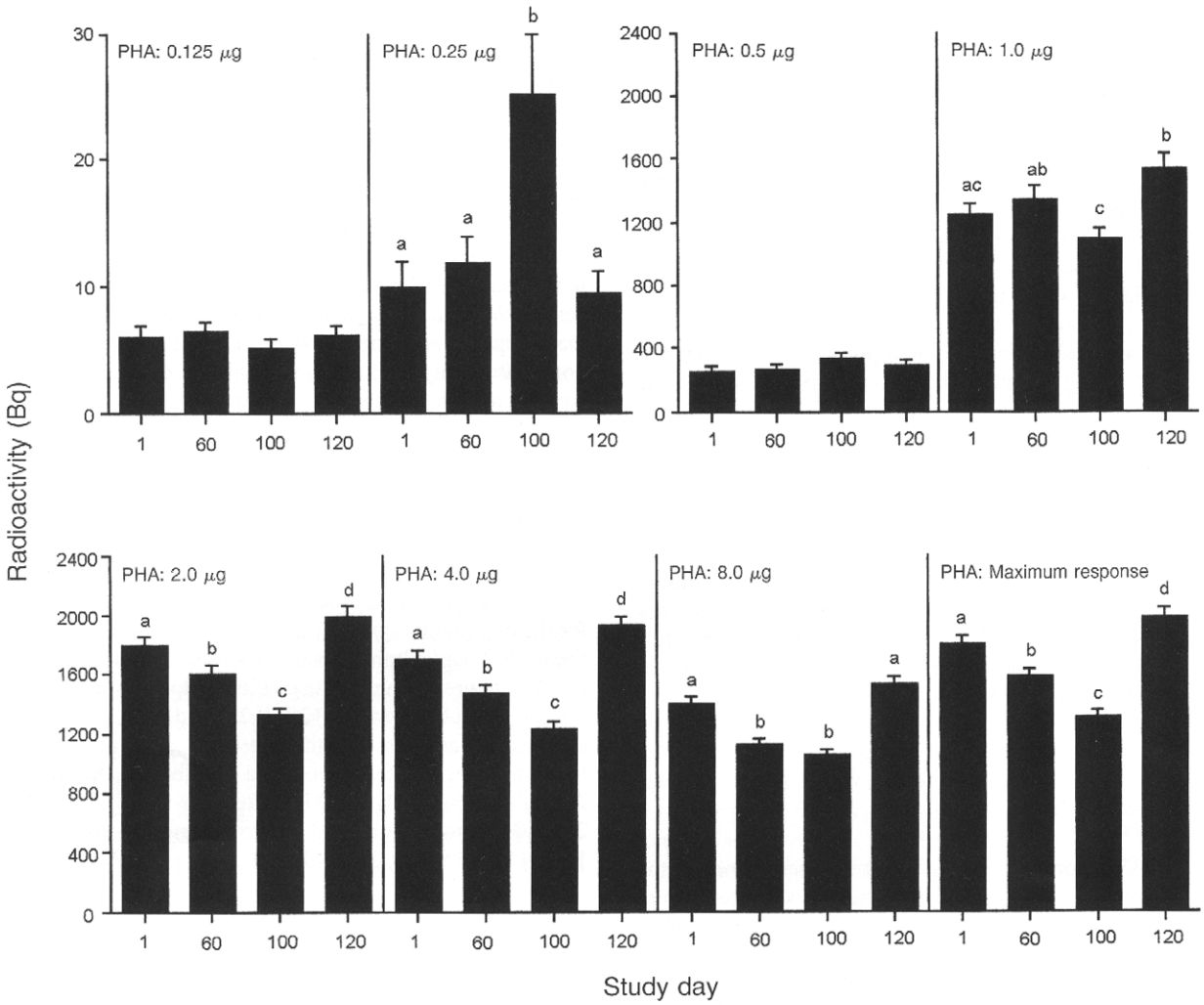

FIGURE 1. Mean (+ SEM) and maximum mitogenic proliferative responsiveness of blood lymphocytes in whole-blood cultures to suboptimal (0.125, 0.25, 0.5, and 1.0 μg/culture), optimal (2.0 and 4.0 μg/culture), and postoptimal (8.0 μg/culture) concentrations of phytohemagglutinin (PHA) in vitro in premenopausal women on a low-carotene diet for 120 d with supplementation with mixed carotenoids during the final 20 d ($n = 9$). Values for the different study days at each concentration of PHA with different superscript letters are significantly different, $P < 0.05$ (statistical analyses described in Methods).

that overnight shipment by air and 24-h delayed setup of whole-blood cultures had little effect on mean maximum mitogenic proliferative responsiveness of blood lymphocytes in vitro to optimal-dose PHA (decreased < 4%) and on mean intersubject CV (increased < 4%). Shipping and delayed processing did, however, have large effects on mean mitogenic proliferative responsiveness of blood lymphocytes in vitro to suboptimal-dose PHA (mean decreases of 45% and 7% in experiments 1 and 2, respectively, with 0.25 μg PHA/culture) and on intersubject CVs (mean increases of 52% and 13% in experiments 1 and 2, respectively). These findings agree with our observation in the present study of larger intersubject CV values for mitogenic proliferative responsiveness of blood lymphocytes in vitro to suboptimal than to optimal doses of PHA. Results of these two experiments suggest that shipment of the blood samples by air and a 24-h delay in processing of the samples had little effect in the present study on maximum mitogenic proliferative responsiveness of blood lymphocytes in vitro to optimal-dose PHA, but did have an effect on suboptimal dose responsiveness. Results from the two experiments also show the need to run dose-response curves when determining the maximum mitogenic proliferative responsiveness of blood lymphocytes in whole-blood cultures in vitro to PHA when the blood samples are scheduled for a 24-h delay in setup.

A second methodologic difference between the present study and that of Daudu et al (18) was the type of cell culture system used to measure the mitogenic proliferative responsiveness of blood lymphocytes in vitro. Daudu et al (18) used density-gradient-prepared PBMCs cultured in nonautologous human serum type AB as the cell culture system. Lymphocyte proliferative responsiveness in the present study was determined in whole-blood cultures, which contain their own natural milieu of unseparated blood components.

Results of the present study and those of van Poppel et al (16) suggest a possible relation between autologous plasma and carotene-modulated lymphocyte proliferation in vitro. van Poppel et al (16) reported enhanced mitogenic responsiveness of lymphocytes cultured in vitro in PBMC preparations containing both nonadherent and adherent cells in autologous plasma from healthy adult male smokers supplemented with 20 mg β-carotene/d for 14 wk. When the autologous plasma was replaced by heterologous fetal bovine serum, they did not

observe the enhancement. Results of the current and a separate unpublished study involving the consumption of carotenoid-rich foods by healthy adults suggest an association between plasma β-carotene concentrations and mitogenic proliferative responsiveness of blood lymphocytes in whole-blood cultures to PHA; however, according to Pearson correlation coefficient analyses, neither study showed a significant association. Studies are in progress to determine whether the association is related to the carotene content of PBMCs.

Using nonadherent PBLs, after removal of adherent lymphoid cells from the PBMC preparation by adherence to plastic, Moriguchi et al (17) showed increased mitogenic proliferative responsiveness of lymphocytes in vitro to optimal-dose PHA (10 mg/L) in young healthy male nonsmokers supplemented with 30 mg β-carotene/d for 30 d. Their results may suggest that the required adherent cell dependence for mitogenic responsiveness of lymphocytes in vitro to optimal-dose PHA is reduced in adult males supplemented with β-carotene. Our use of whole-blood cultures in the present study for lymphocyte proliferation in vitro prevented us from addressing the role of adherent cells on carotene-modulated mitogenic proliferative responsiveness. Future studies need to examine this possible relation. Results of the present study show that a diet low in carotene has a suppressive effect on the maximum mitogenic proliferative responsiveness of immune system blood lymphocytes in vitro to optimal-dose PHA. We also showed that the suppression is not corrected by supplementation with low-dose β-carotene, but is corrected with concentrates from vegetables rich in carotenoids, even though the amount of β-carotene received from the mixed carotenoid concentrates is relatively small compared with most studies involving supplementation of humans with isolated β-carotene. Because of this and our observed lack of a significant relation between serum β-carotene concentrations and lymphocyte proliferation in vitro, future studies need to evaluate the role of other phytonutrients missing from a low-carotene diet or present in concentrates of vegetables, which may have a modulating effect on immune cell lymphocyte function.

We are thankful for the technical assistance of Mary Pat Howard and Terry R Neidlinger, for carotenoid analysis of the mixed-carotenoid capsule by CMM Smith, and for the statistical expertise of Jesse Chittam.

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