β-Carotene-induced enhancement of natural killer cell activity in elderly men: an investigation of the role of cytokines1–4

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ABSTRACT We showed previously that natural killer (NK) cell activity is significantly greater in elderly men supplemented with β-carotene than in those taking placebo. In an attempt to determine the mechanism of β-carotene’s effect, we analyzed the production of NK cell–enhancing cytokines (interferon α, interferon γ, and interleukin 12). Boston-area participants in the Physicians’ Health Study (men aged 65–88 y; mean age, 73 y) who had been supplemented with β-carotene (50 mg on alternate days) for an average of 12 y were enrolled in a randomized, placebo-controlled, double-blind study. Elderly subjects taking β-carotene supplements had significantly greater plasma β-carotene concentrations than those taking placebo. β-Carotene–supplemented elderly men had significantly greater NK cell activity than did elderly men receiving placebo. Percentages of NK cells (CD16⁺CD56⁺) were not significantly different between the β-carotene and placebo groups. Production of interleukin 12, interferon α, or concanavalin A–stimulated interferon γ by cultured peripheral blood mononuclear cells was not significantly different between β-carotene–supplemented elderly and those taking placebo. Our results indicate that β-carotene-induced enhancement of NK cell activity is not mediated by changes in percentages of CD16⁺CD56⁺ NK cells nor through up-regulation of interleukin 12 or interferon α. Am J Clin Nutr 1998;68:164–70.

KEY WORDS Natural killer cell activity, interferon α, IFN-α, interferon γ, IFN-γ, interleukin 12, IL-12, β-carotene, aging, elderly men, cytokines, Physicians’ Health Study

INTRODUCTION

Evidence suggests multiple roles for natural killer (NK) cells in the maintenance of health, including antitumor activity (1). NK cells also exhibit immunoregulatory activities, such as playing an accessory role in the induction of cytotoxic T cells (2) and producing cytokines [interferons (IFNs)] important in antiviral defense (3). In addition, NK cells produce chemokines, such as macrophage inflammatory protein 1α, which can augment NK cell activity and may contribute to control of infection and inflammation (4). Recent research on the mechanisms of NK cell activity has clearly shown significant amounts of NK cell–mediated killing in a variety of solid tumors (via the nonsecretory, apoptotic pathway) and leukemias (via the secretory, necrotic pathway), suggesting a role for NK cells in immunosurveillance and control of cell growth (5).

In a previous study, we showed that NK cell activity was enhanced in elderly men who had been supplemented with β-carotene for an average of 12 y when compared with men who had taken placebo (6). This β-carotene–induced enhancement was not due to an increase in the percentage of CD16⁺ NK cells, the production of interleukin 2 (IL-2), the up-regulation of IL-2 receptor expression, or a decrease in prostaglandin E₂ production (6). It is possible, however, that other cytokines, such as IL-12 or IFN-γ, whose activity is independent of IL-2 and is largely cytotoxic rather than proliferative (7), could be up-regulated by β-carotene to increase NK cell activity.

Production of IL-12, or NK cell stimulatory factor, as it is also called, by monocytes and macrophages leads to up-regulation of IFN-γ production by NK cells (8) and enhancement of NK cell cytotoxicity (9). Addition of IL-12 to aged murine cells augmented their weak cytotoxic T lymphocyte activity to levels comparable with those in young mice (10). IL-12 production in elderly humans has not been reported previously.

IFNs of type I (IFN-α and IFN-β) and type II (IFN-γ) significantly enhance production of NK cell cytotoxic factors in secretory granules that up-regulate target cell lysis; this enhancement is significantly decreased by the addition of neutralizing antibodies (11). Most studies agree that production of IFN-α...
(12–14) and IFN-γ (12, 13) is significantly depressed in the elderly, although one study reported no change in IFN-γ production with age (14).

The purpose of this study was to determine whether the β-carotene–induced enhancement of NK cell activity in the elderly is mediated through up-regulation of one or more NK cell–stimulatory cytokines, ie, IFN-α, IFN-γ, or IL-12, as these cytokines have been shown to modulate different stages of NK cell cytotoxicity and may be produced in lower amounts in aging individuals.

SUBJECTS AND METHODS

Study population

The Physicians’ Health Study is a randomized, double-blind, placebo-controlled trial including 22,071 US male physicians that is testing low-dose aspirin and β-carotene (as Lutein; BASF, Mount Olive, NJ; 50 mg on alternate days) supplementation in the primary prevention of cardiovascular disease and cancer. With use of a 2 × 2 factorial design, subjects were randomly assigned to receive aspirin, β-carotene, both, or neither between 1982 and 1984. The randomized aspirin component was terminated in December 1987 by the external Data Monitoring Board primarily because of a highly significant 44% reduction in risk of a first myocardial infarction in the aspirin group (15). The randomized β-carotene component continued uninterrupted until it ended as scheduled on December 31, 1995, representing an average of > 12 y of treatment and follow-up.

A random sample of highly compliant Boston-area participants in the Physicians’ Health Study was recruited for this substudy because the immunologic tests required two fresh blood samples from each subject. Samples were collected from August through November of 1995. This project was approved by the Human Investigation Review Committee of Tufts University Health Sciences and New England Medical Center Hospitals and the approval was acknowledged by the Internal Review Board of Brigham and Women’s Hospital and Harvard Medical School. Informed, written consent was obtained from all participating physicians.

Recruitment was done by telephone by investigators blinded to treatment assignments, with the goal of enrolling 20 non-smoking physicians from each coded treatment group. At the time of recruitment, subjects were asked to refrain from taking multivitamin supplements containing vitamins in amounts near the recommended dietary allowance (16) for ≥ 1 mo before the scheduled blood draws; subjects who could not meet this criterion were excluded. Subjects known to be taking immunomodulatory drugs and those participating in extensive athletic training were excluded during recruitment to avoid confounding data results. Participating physicians were asked to refrain from taking aspirin or other nonsteroidal antiinflammatory drugs for 7 d before the blood draw.

Because of the nature of the study, physicians were admitted based on their willingness to collaborate as a part of the Physicians’ Health Study cohort. The population was considered healthy at baseline (at entrance to the Physicians’ Health Study between 1982 and 1984), with no reported history of myocardial infarction, stroke, angina pectoris, cancer, or other major illnesses. In addition, at the time of the substudy in 1995, 46% took no regular medications, 41% took 1–2 medications, and 13% took ≥ 3 medications, the majority being antihypertensive agents and cholesterol-lowering drugs. Participants who had reported a major cardiovascular or cancer event were excluded from this substudy. According to on-site questionnaires completed at the time of the blood draw, the physicians were in general good health.

Blood samples from fasted subjects were collected at the physicians’ residence or place of employment between 0600 and 0930. Samples were collected from each group randomly across the given time period. To avoid differences in immunosensitivity between subjects, the subjects were asked to refrain from receiving influenza vaccines until after their second blood draw, at which time we administered the vaccine if desired.

Compliance with study supplement intake for 2 mo before blood collection was self-reported as part of the questionnaire. This method of compliance assessment was validated previously by comparison with blood concentrations. The sample for the current study was highly compliant: 72% of the subjects reported taking all of their pills, 15% missed 1–3 pills, and 8% missed 4–9 pills; those missing 10–15 pills (≥ 3 wk of supplementation) were excluded from the data analyses. Five participating physicians were excluded from the data analyses: 2 because of poor compliance and 3 because of use of nonsteroidal antiinflammatory drugs within 72 h of the blood draw.

Plasma β-carotene analysis

Plasma β-carotene, retinol, and α-tocopherol concentrations were measured with reversed-phase gradient HPLC equipment and procedures described previously (17).

Mononuclear cell isolation

Blood from fasted subjects was collected in foil-wrapped, evacuated tubes containing sodium heparin and was kept at room temperature in the dark until processed. All laboratory procedures were conducted under red light to minimize decomposition of β-carotene from ultraviolet light exposure. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density-gradient centrifugation. The mononuclear cell “buffy layer” was isolated and washed thrice in complete media: RPMI 1640 (BioWhittaker, Walkersville, MD) with 100 mmol L-arginine/L (Gibco BRL, New York), 2 mmol glutamine/L (Gibco BRL), and 25 mmol HEPES/L (Sigma, St Louis). Cell viability was assessed by trypan blue staining and PBMCs were counted in a hemocytometer under a light microscope.

Determination of percentages of NK cells

Percentages of NK cells were determined by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) on doubly stained PBMCs. All procedures were carried out at 4 °C and were modified for double staining from the previous description (18). Briefly, 5 × 10⁷ PBMCs were stained with optimal concentrations of fluorescence-tagged monoclonal antibodies: Leu 11c, anti-CD16, fluorescein, and Leu 19, anti-CD56, phycoerythrin (Becton Dickinson). Preliminary experiments showed no cross-reactivity between antibodies; therefore, stains were added in series and allowed to bind for 30 min. Stained cells were washed in cold phosphate-buffered saline (0.1% sodium azide and 2% fetal bovine serum) and were fixed in 0.5% paraformaldehyde in preparation for acquisition and analysis.
NK cell activity

The procedure for determination of NK cell activity was modified slightly from the previous description (6). Briefly, K562 human myelogenous leukemia cell targets (kindly provided by J Lieberman, Tufts University, Boston) were labeled with $^{51}$Cr (25 μL × decay factor/10 × 10^8 cells; DuPont NEN, Boston) and incubated for 60–90 min. Targets [1 × 10^6/well in 96-well, round-bottomed plates (NuncIOn; Nunc, Roskilde, Denmark)] were added to appropriately diluted PBMCs (1 × 10^6 cells/L) for final effector to target cell ratios of 3.12:1, 6.25:1, 12.5:1, 25:1, 50:1, and 100:1. Dilutions were plated in triplicate and cells were allowed to incubate for 4 h. Spontaneous release was measured as the amount of $^{51}$Cr released by target cells alone (6 wells) and maximum release was measured by the amount of $^{51}$Cr released through three cycles of freezing (−70 °C) and thawing (24 °C). Supernates were spotted onto thick mats and sealed in plastic bags for gamma counting in a chromium window on the 1205 BetaPlate liquid scintillation counter (Wallac, Turku, Finland). Spontaneous release remained below 10% with the exception of one occasion during which it increased slightly to 15%. Percentage specific lysis was defined as follows:

Specific lysis (%) =

\[
\text{Specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100
\]

(1)

where cpm is counts per minute.

To reduce the NK cell cytotoxicity data to a single value that was meaningful in terms of lytic activity, lytic units, the number of PBMCs required to cause 30% target cell lysis, were calculated. Data from the four lowest effector to target cell ratios were reduced to linearity and lytic units were defined by a simple exponential fit equation (19):

\[
y = 1 - e^{-kx}
\]

(2)

where y is cell-mediated $^{51}$Cr release, x is the effector to target cell ratio, and k is the slope obtained by plotting the logarithm (A − y), where A = 100, the maximum of the dose-response curve.

Cytokine production

IFN-α was induced from PBMCs by a double-stranded RNA virus, murine encephalomyocarditis virus (ATCC, Rockville, MD), at a 1:1600 dilution of original stock [titer 10(6.75)]. Cultures were prepared in quadruplicate by plating 1.0 × 10^6 cells/L [96-well, round-bottomed plates (Nunclon, Nunc)] in 10% autologous plasma.

IFN-γ and IL-12 cultures were prepared by plating 1.0 × 10^9 cells/L [24-well, flat-bottomed plates (Falcon; Becton Dickinson and Co, Lincoln Park, NJ)] in 10% autologous plasma. PBMCs were stimulated with 10 ng concanavalin A/L (Sigma) for production of IFN-γ (major source; T cells) and with 0.0075 wt/vol *Staphylococcus aureus* Cowan 1 ([SAC] Zysorbin; Zymed, San Francisco) for measurement of IL-12 and IL-12–induced IFN-γ (major source: NK cells) (8). All cultures were incubated for 24 h in 5% carbon dioxide at 37 °C, after which cell-free supernates were harvested and frozen at −20 °C until analyzed.

Cytokines were analyzed by using enzyme-linked immunosorbent assays as detailed below. Stimulated supernates were analyzed in duplicate along with appropriate unstimulated controls. Colorimetric readings were recorded at 450 nm with a Bio-Tek EL 340 Bioelectronics Reader (Bio-Tek Instruments, Winooski, VT) and calculations of cytokine concentrations were made with DELTASOFT II (version 4.1 for Macintosh, Bio-Tek Instruments).

Interferon-α

IFN-α was measured with the Cytoscreen Immunoassay Kit for human IFN-α (Biosource International, Camarillo, CA). The kit was a sandwich immunoassay that consisted of 96-well plates precoated with a monoclonal antibody specific for IFN-α (and having no cross-reactivity with IFN-β or IFN-γ). The anti-secondary antibody was conjugated to horseradish-peroxidase, with tetramethylbenzidine as the substrate. The human IFN-α international reference standard was provided by the National Institutes of Health (reference no. Gxa01-901-535) (20). The range of the assay was between 0 and 500 ng/L and the sensitivity of the assay was < 25 ng/L.

Interferon-γ

IFN-γ was measured with the Cytoscreen Immunoassay Kit for human IFN-γ (Biosource International). The kit was a sandwich immunoassay that consisted of 96-well plates precoated with a monoclonal antibody specific for IFN-γ (< 0.05% cross-reactivity with IL-4, IL-6, IL-10, tumor necrosis factor α (TNF-α), and colony-stimulating factor). The IFN-γ binds simultaneously to the capture antibody and to the polyclonal biotin-conjugated secondary antibody. The four-member sandwich is completed by the addition of streptavidin-peroxidase, for which tetramethylbenzidine served as the substrate. The range of the assay was between 0 and 1000 ng/L and the sensitivity of the assay was < 4 ng/L.

Interleukin-12

IL-12 was measured with the Quantikine Human IL-12 Immunoassay (R&D Systems, Inc, Minneapolis). The kit was a sandwich immunoassay that consisted of 96-well plates precoated with a monoclonal antibody specific for the IL-12 heterodimer (not the individual subunits of the dimer; no cross-reactivity with 68 human cytokines and receptors). The secondary polyclonal antibody to IL-12 was conjugated to horseradish-peroxidase, with tetramethylbenzidine as the substrate. The human recombinant IL-12 standard was expressed in S21 insect cells by using a baculovirus expression system (R&D Systems). The range of the assay was between 0 and 500 ng/L and the minimum detectable dose was 5.0 ng/L.

Statistics

The a priori sample size calculations were based on the β-carotene group mean for the NK cell activity of elderly men being 15.4 units greater than activity in the placebo group. This estimated increase was based on an earlier substudy of Boston-area participants in the Physicians’ Health Study (6). The within-group SD was 14 units (6). For a sample size of 20 subjects per group, the probability of detecting a difference at the 0.05 level of significance was calculated to be 0.924; for a sample size of 14 subjects per group, the probability was calculated to be 0.800 (PC SIZE: CONSULTANT, version 1.01, by Gerard E Dallal, 1990).

Data that were not normally distributed were log-transformed for all statistical analyses and subsequently transformed back for reporting in standard units; 95% CIs are given for the geometric
Data were analyzed by using unpaired Student’s t tests (SYSTAT for the Macintosh, version 5.2; SYSTAT, Evanston, IL). Correlations were calculated by using Pearson’s correlation coefficients and Bonferroni adjustments for multiple comparisons were made when necessary. Significance was defined by a P value ≤ 0.05. Unless stated otherwise, results are reported as means ± SEMs.

RESULTS

Subject characteristics are shown in Table 1. There were no significant differences in mean age or body mass index between treatment groups. β-Carotene concentrations in the supplemented group were elevated more than fourfold over those in the placebo group (P < 0.00001). No significant differences in plasma retinol or α-tocopherol concentrations were present between the β-carotene and placebo groups.

Consistent with previously reported findings (6), NK cell activity, expressed as percent cytotoxicity and standardized in lytic units of activity, was significantly greater in the elderly supplemented with β-carotene than in subjects taking placebo (Table 2). This enhancement of NK cell activity occurred without a significant difference in the percentage of NK cells between treatment groups (Table 2).

The production of multiple cytokines by PBMC cultures from those taking placebo and those supplemented with β-carotene is shown in Table 3. IFN-γ production was induced by two distinct stimuli: the lectin concanavalin A, which induces IFN-γ primarily from T cells, and SAC, which induces secondary production of IFN-γ by NK cells in response to IL-12. Amounts of IFN-γ produced as a result of either stimuli were not significantly different between β-carotene–supplemented and placebo groups, although a trend was observed for greater production of SAC-stimulated IFN-γ in the β-carotene–supplemented group. Note that sample collection for the analysis of SAC-stimulated IFN-γ began after the substudy was initiated. For this reason, sample sizes were smaller for SAC-stimulated IFN-γ (placebo group, n = 14; β-carotene group, n = 10) than for the other cytokines. This increases the type II error, which indicates that a potential enhancing role for SAC-stimulated IFN-γ in the up-regulation of NK cell activity in the β-carotene group may not have been detectable.

IL-12 production was not significantly up-regulated by β-carotene compared with placebo, although cell cultures from some β-carotene–supplemented individuals tended to produce more IL-12 than did cell cultures from individuals taking placebo. No significant differences between the β-carotene and placebo groups were found in production of IFN-α in response to viral challenge of PBMCs in culture, although production of IFN-α tended to be greater in those supplemented with β-carotene.

A weak, significant, positive correlation (r = 0.36, P = 0.05) between plasma β-carotene concentrations and NK cell lytic activity was present (Figure 1); this association supports the up-regulation of NK cell activity seen among β-carotene–supplemented elderly. Analyses of correlations between cytokine production and lytic activity of NK cells revealed a strong, significant, positive correlation between IL-12 production and lytic activity (r = 0.82, P = 0.003), underscoring the importance of this cytokine in NK cell–mediated killing of tumors in this culture system. A marginally significant positive correlation between SAC-stimulated IFN-γ production (by NK cells via IL-12) and NK cell lytic activity (r = 0.59, P = 0.06) was also observed; however, Bonferroni adjustments for comparing associations of multiple cytokines to NK cell activity further removed this association from significance. No significant correlations were observed for associations between plasma β-carotene concentrations and production of individual cytokines.

DISCUSSION

On two separate occasions (1993 and 1995) we showed that elderly men supplemented with β-carotene for an average of 12 y had significantly greater NK cell activity, in the absence of differences in percentages of NK cells, than did persons taking a placebo. In an effort to reveal the mechanism of this age-specific

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age</th>
<th>BMI</th>
<th>Plasma β-carotene</th>
<th>Plasma retinol</th>
<th>Plasma α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo (n = 20)</td>
<td>74 ± 2</td>
<td>24.8 ± 0.6</td>
<td>0.47 (0.2, 1.0)</td>
<td>2.07 ± 0.09</td>
<td>28.4 ± 1.4</td>
</tr>
<tr>
<td>β-Carotene (n = 14)</td>
<td>72 ± 2</td>
<td>24.4 ± 0.9</td>
<td>2.31 (1.1, 5.1)²</td>
<td>2.24 ± 0.09</td>
<td>27.2 ± 1.7</td>
</tr>
</tbody>
</table>

1 ± SEM, except plasma β-carotene, which is geometric (95% CI of x).
2 Significantly greater than placebo, P < 0.000001 (unpaired t test on log-transformed data).

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of NK cells</th>
<th>Ratio of effector to target cells</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>3.12:1</td>
<td>6.25:1</td>
</tr>
<tr>
<td>Placebo (n = 17)</td>
<td>16.1 ± 1.8</td>
<td>11.5 ± 1.8</td>
<td>17.7 ± 2.5</td>
</tr>
<tr>
<td>β-Carotene (n = 13)</td>
<td>17.5 ± 2.5</td>
<td>18.8 ± 3.2³</td>
<td>28.2 ± 3.9⁰</td>
</tr>
</tbody>
</table>

1 ± SEM.
2 Reflects the percentages of CD16⁺CD56⁺ NK cells from 5000 peripheral blood mononuclear cells acquired by flow cytometry.
3 Percent specific lysis = (experimental cpm - spontaneous cpm/maximum cpm - spontaneous cpm) × 100, where cpm is counts per minute.
4 Lytic unit (LU) = the number of peripheral blood mononuclear cells required to cause 30% target cell lysis.
5 Significantly greater than placebo, P ≤ 0.05.


TABLE 3
Effect of β-carotene supplementation on production of cytokines by peripheral blood mononuclear cells

<table>
<thead>
<tr>
<th>Cytokine (stimulus)</th>
<th>Treatment</th>
<th>IFN-γ (ConA)</th>
<th>IFN-γ (SAC)</th>
<th>IL-12 (SAC)</th>
<th>IFN-α (EMCV)</th>
</tr>
</thead>
</table>

**Note:** *T ± SEM, except IFN-α, which is geometric T (95% CI of T); α in brackets. Peripheral blood mononuclear cells were cultured in 10% autologous plasma for 24 h and quantified by enzyme-linked immunosorbent assay. IFN, interferon; ConA, concanavalin A; SAC, Staphylococcus aureus Cowan I; IL, interleukin; EMCV, murine encephalomyocarditis virus.

β-carotene–induced enhancement of NK cell activity, we investigated the ability of PBMCs from individuals in each treatment group to produce a variety of soluble mediators that have been shown to play a role in NK cell cytotoxicity. To our knowledge, the effect of β-carotene on production of these cytokines in the elderly has not been reported previously.

Production of IL-12 and IFN-α and production of IFN-γ by T cells (with concanavalin A stimulation) and by NK cells (with SAC stimulation) were not significantly up-regulated as a result of long-term β-carotene supplementation. However, because of the smaller sample size in the analysis of SAC-stimulated IFN-γ, this study does not provide sufficient evidence of the absence of a role for this particular cytokine in the enhancement of NK cell activity in elderly men supplemented with β-carotene. Analysis of archived 48-h concanavalin A–induced supernates from the 1993 substudy (6) also showed no significant difference in IFN-γ production between the β-carotene (3.3 ± 0.9 μg/L) and placebo groups (2.9 ± 0.6 μg/L). Experiments investigating the effects of in vitro β-carotene supplementation on the production of IFN-γ support our in vivo experiments; no significant increase in IFN-γ production stimulated by antigen priming was seen after in vitro β-carotene supplementation of murine cells (21), and in vitro β-carotene supplementation of human PBMCs resulted in no significant difference in the production of phytohemagglutinin-stimulated IFN-γ between supplemented cells and β-carotene–free liposome controls (22).

In a previous report describing the 1993 substudy (6), we were able to rule out the possible effect of β-carotene on enhancing NK cell activity in the elderly through a decrease in the production of prostaglandin E2, a suppressor of NK cell activity. In addition, we showed previously that long-term β-carotene supplementation did not result in up-regulation of the production of IL-2, a cytokine important in stimulating the proliferation of NK cells (6). In conjunction with this finding there were no differences in the percentage of CD16+ NK cells between treatment groups (6).

Although insufficient cell numbers did not allow for a thorough analysis of TNF-α production in the present study, we were able to analyze a subgroup of persons aged 51–86 years from the 1993 substudy. We found significantly enhanced lipopolysaccharide-induced TNF-α production in β-carotene–supplemented subjects (11.4 ± 1.9 μg/L; n = 7) compared with those given placebo (5.64 ± 0.56 μg/L; n = 9) (P = 0.03). Although this group included subjects younger than those in the current study (≤65 years), it is possible that increased TNF-α production as a result of β-carotene supplementation may have contributed to the observed enhancement in NK cell activity. Cytotoxic lymphokines such as TNF-α may gain entry to the target through pores made by perforin exocytosed from NK cells, without the need for receptor-mediated entry, where they could contribute to degradation of target cell DNA (23). It has been shown histologically that β-carotene–induced production of TNF-α by macrophages (achieved with topical and injected forms of β-carotene) was at least partially responsible for the regression of established hamster buccal pouch carcinomas (24). In addition, patients with oral leukoplakia who were responsive to β-carotene treatment were found to have significantly higher plasma concentrations of TNF-α than patients who did not respond to β-carotene treatment (26). Furthermore, in vitro addition of β-carotene (1 × 10^-8 to 1 × 10^-7 mol/L in liposomes) to PBMCs resulted in a significant increase in the release of TNF-α when compared with β-carotene–free liposome controls (22).

Recent research has shown that co-stimulation of human NK cells with IL-12 and IL-15 results in a synergistic up-regulation of the production of macrophage inflammatory protein 1α, which, when added to NK cells in vitro, results in up-regulation of NK cell cytotoxicity (4). Although IL-15 and macrophage inflammatory protein 1α were not measured in our study, data from Bluman et al (4) raise the possibility of synergism between cytokines that act to co-stimulate and subsequently up-regulate NK cell activity. It may be possible that small increases in the production of multiple cytokines (SAC-stimulated IL-12 and...
IFN-γ or IFN-α or both) by cell cultures of β-carotene-supplemented individuals may result in additive or synergistic effects that could contribute to significant up-regulation of NK cell activity in the absence of statistically significant differences in the production of individual cytokines.

Another mechanism that might lead to enhancement of NK cell cytotoxicity by β-carotene is up-regulation of cellular lytic events. Up-regulation of signaling events important to NK cell activation, such as calcium uptake (flux) or activation of protein kinase C, may up-regulate apoptotic or membrane-lytic target cell death (5). Up-regulation of perforin or granzyme production at the message or protein levels or up-regulation of granzyme activity may lead to faster target cell lysis (23, 27). These possible mechanisms remain to be explored.

Retinoic acid has been characterized by its active role in gene regulation (28) and has been shown in ferrets to be a metabolite of β-carotene (29). In vitro addition of retinoic acid to non-adherent murine splenocytes cultured with IL-2 resulted in significant augmentation of lymphokine-activated killer cell activity, which was positively correlated with increased serine protease activity (30) and later shown to increase perforin expression at both the message and protein levels (31). However, neither dietary nor supplemental β-carotene in humans resulted in significantly greater retinoic acid concentrations in plasma or adipose tissue, although the authors suggested that larger studies be carried out to determine with greater clarity whether concentrations of adipose retinoic acid increase in response to β-carotene supplementation (32). Although plasma retinol concentrations tended to be greater in the β-carotene group, there were no significant differences in plasma retinol between the β-carotene and placebo groups; this finding is in accord with results published previously (6, 33). Concentrations of plasma or tissue retinoic acid were not measured in our study; therefore, a specific role for retinoic acid cannot be determined.

This report confirms that long-term β-carotene supplementation significantly enhances NK cell activity in elderly men when compared with placebo, in the absence of differences in percentages of NK cells. This enhancement in NK cell activity was not explained by up-regulation of individual NK cell–stimulatory cytokines such as IFN-α, concanavalin A–stimulated IFN-γ, IL-12, or IL-2 (6), nor by down-regulation of the NK cell–suppressive factor prostaglandin E₂ (6). Additional studies with larger sample sizes are needed to establish a clear role for SAC-stimulated IFN-γ in relation to the enhanced NK cell activity in those supplemented with β-carotene. Although the exact mechanisms have not been delineated, potential up-regulation of TNF-α or synergism between cytokines that act to co-stimulate NK cells and subsequently up-regulate NK cell activity might contribute to the higher NK cell activity of β-carotene–supplemented elderly men.

We express our sincere appreciation to all participating physicians from the Physicians’ Health Study, without whose dedicated collaboration this project would not have been feasible. Gratitude is also extended to the nursing staff of the Metabolic Research Unit for their participation in our mobile sample collection, to Keith Martin and Mohsen Meydani for their assistance with HPLC procedures, and to Christine A Biron (Brown University) for her guidance and expertise in the influence of cytokines on natural killer cell activity.

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