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ORIGINAL RESEARCH

IMMUNE FUNCTION IN ELDERLY SMOKERS AND NONSMOKERS IMPROVES DURING SUPPLEMENTATION WITH FRUIT AND VEGETABLE EXTRACTS

PAULA F. INSERRA, PHD, SHUGUANG JIANG, BM, DAVID SOLKOFF, BS, JEONGMIN LEE, MS, ZHEN ZHANG, PHD, MINJAN XU, PHD, ROBERT HESSLINK, JR., PHD, JOHN WISE, PHD, AND RONALD R. WATSON, PHD

Background: Epidemiological evidence suggests fruits and vegetables reduce the risk of cardiovascular disease (CVD) and cancer. Immune function declines with age as CVD and cancer incidence rises and may be related to poor antioxidant status.
Objective: To investigate how fruit and vegetable extracts (Juice Plus™) containing multiple antioxidants and phytonutrients affect immune function in the elderly.
Design: Subjects (n = 53; aged 60–86 years, mean = 68 years) consumed extracts for 80 days and two blood samples were taken at baseline and then one at days 40 and 80.
Results: Significant increases were found in the serum antioxidants when baseline values were compared with day 80; lutein/zeaxanthin ($p < .005$), α-carotene ($p < .0001$), β-carotene ($p < .0001$), lycopene ($p < .05$), and α-tocopherol ($p < .005$). Spontaneous proliferation of PBM cells increased significantly ($p < .0001$). Natural killer (NK) cell cytotoxicity significantly increased at effector to target cell ratios of 100:1 ($p < .0001$), 50:1 ($p < .0005$), and 25:1 ($p < .005$). Supernatant from PBM cells stimulated with phytohemaglutinin (PHA; 10 μg/mL) resulted in significant twofold increases in interleukin-2 (IL-2) ($p < .0001$). Additionally, statistically significant increases in IL-2 production were observed in smokers ($p < .005$).
Conclusions: Fruit and vegetable extract supplementation significantly enhanced multiple measures of immune function in elderly subjects, and improved IL-2 levels in smokers. Fruit and vegetable extract supplementation offers a novel way to improve compliance with current nutritional recommendations and may potentially lower disease risk. (Int Med 1999;2:3–10) © 1999 Elsevier Science Inc.

Key Words: carotenoids; α-tocopherol; fruits and vegetables; immune function; aging; smoking.

The incidence of cardiovascular disease (CVD) and cancer rises dramatically with age as immune function declines. Recently, development of atherosclerosis has been attributed to the oxidation of low-density lipoprotein (LDL) [1]. The imbalance of antioxidants and free radicals may be a contributing factor that further impairs immune function in the aged and leads to an inappropriate induction of the inflammatory response. Preventing immune damage with antioxidants may be a way to decrease CVD risk. Additionally, initiation of cancer cells may also be a result of free radical damage and poor antioxidant status. Natural killer (NK) cells are capable of eliminating cancer cells. Impaired NK cell cytotoxicity in the elderly may be another event that increases cancer risk.

Epidemiological evidence suggests a beneficial role of dietary fruits and vegetables in reducing risk for cancer and CVD. The observed inverse relationships between fruit and/or vegetable consumption and lung cancer [2,3], colorectal cancer [4], total cancer mortality [5], and CVD [6] have been attributed to dietary antioxidants such as β-carotene and vitamin E. Interestingly, no decrease in the incidence of disease has been observed with supplemental β-carotene [7] and, in fact, increased risk of lung cancer was observed in smokers [8,9]. Whether these studies

From the Arizona Prevention Center (Inserra, Jiang, Solkoff, Lee, Zhang, and Watson) and the Arizona Cancer Center (Xu), University of Arizona, Tucson, Arizona, and Natural Alternatives International, San Marcos, California (Hesslink Jr. and Wise).

Address reprint requests to: Ronald R. Watson, Arizona Prevention Center, PO Box 245155, University of Arizona, Tucson, AZ 85724, USA; Tel: (520) 626-2850; Fax: (520) 626-6093; E-Mail: rwatson@u.arizona.edu.

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failed to supplement with the optimal dosage, or whether other protective factors [10] are present in fruit and vegetables that work alone or in combination with antioxidants, such as β-carotene, remain to be answered. However, there is no reason to believe that essential antioxidant vitamins are the only constituents in plants that have health-promoting benefits. Therefore, we tested the benefits of the small molecular weight materials in fruits and vegetables in overcoming immune dysfunction. Vitamin E has been shown consistently to improve cellular immunity in rats and mice [11–13]. Similarly, β-carotene increased the in vitro stimulation of mouse splenocytes [14]; however, the evidence is not as convincing for humans [15,16]. Therefore, current public health messages encourage increasing consumption of fruit and vegetables [17] to at least five servings per day instead of promoting supplement use.

Unfortunately, consumption has not changed [18], despite such strong recommendations, and is particularly low among adolescents [19] and patients with increased risk of colorectal cancer [20]. Furthermore, large discrepancies have been seen with self-reported and actual fruit and vegetable intake, as individuals tend to overestimate their actual intake [21]. Thus, nutritional recommendations tend to be ineffective [21]. We have therefore used fruit and vegetable extracts to supplement dietary intake. Because increasing consumption of fruits and vegetables tends to be difficult, perhaps supplemental extracts could be a way to provide some, or all, of the benefits supplied by fruits and vegetables themselves.

The aim of the current study was to determine the effect supplementation with fruit and vegetable extracts on various measures of immune function among smokers and nonsmokers. As our supplements helped overcome immmunosenescence, our study shows the immune benefits of intakes of soluble materials from a broad range of plant materials. The data we found in the present study encourage greater consumption of a variety of fruits and vegetables by aging adults to promote improved immune defenses.

SUBJECTS AND METHODS

Subjects

Fifty-five subjects over the age of 60 years were recruited for the 3-month study. Subjects were recruited from a database of participants in previous studies. This database comprised approximately 200 healthy men and women over the age of 60 years. Additional subjects were recruited from an advertisement in a newspaper from a local retirement community. Subjects were screened for past medical history, alcohol consumption, and smoking status. Subjects over the age of 60 years were eligible for participation. Subjects with known active cancers or uncontrolled diabetes were not eligible for participation. A total of 46 subjects completed the study. Nine individuals dropped out during the course of the study period; 4 subjects moved away from the area, 2 subjects developed a hive-like rash, and 3 subjects decided not to continue (Table 1). Data obtained from subjects who dropped out after day 40 of treatment were included in the results. Because only 2 individuals dropped out before day 40, data from a total of 53 subjects were available for use in the final analysis. Blood samples were taken two separate days over 1 week before supplementation (visits 1 and 2) and two samples were taken during the supplementation period (once after 40 days of supplementation and one after 80 days; visits 3 and 4). The study was approved by the Human Subjects Committee of the University of Arizona and all subjects provided written informed consent prior to their participation.

Table 1. Subject's characteristics

Characteristic	Men ($n = 21$)	Women ($n = 32$)
Smoking status		
No	18	22
Yes	3	10
Age distribution (years)		
60–65	10	15
66–70	2	7
71–75	5	5
76–80	4	3
>80	0	2
Medical history		
Hypertension	4	9
Heart disease	8	6
Hypothyroid	0	8
Diabetes (type II)	5	3
Arthritis	0	3
Other (ulcer, pulmonary disease)	3	2
Hormone replacement	N/A	13
Vitamin/mineral supplementation	12	18
Average BMI	23.97	23.89

BMI, body mass index.
Available data from 53 subjects were used in the analysis. A total of 9 subjects dropped out of during the course of the study. Two subjects dropped out before day 40 of treatment and these data were excluded. The 7 remaining subjects dropped out after day 40 and these data were used in the final analysis. Of the 7 subjects, 2 were male nonsmokers, 1 was a male smoker, 3 were female nonsmokers, and 1 was a female smoker.

During visits 2 and 3, subjects were given a supply of supplements to last until their next visit. Subjects were instructed to consume 2 fruit capsules in the morning with an 8-oz glass of water and a meal and 2 vegetable capsules in the evening with an 8-oz glass of water and a meal. This information was emphasized verbally and in writing at visits 2 and 3. Written information was provided as a label on the supplements. Subjects were otherwise instructed to eat as they normally would. Subjects were also instructed to bring any remaining pills to the clinic at day 40 and day 80, at which time pill counts were conducted.

Supplements

The fruit and vegetable extracts were obtained from NSA International (Memphis, TN) and consisted of dried fruit

and vegetable powders prepared as described previously [22]. Briefly, fruit juice supplements contained 850 mg fruit powder per capsule, made of extracts from apples, oranges, pineapples, papaya, cranberries, and peaches. Vegetable supplements contained 750 mg vegetable powder per capsule and contained extracts from carrots, parsley, beets, broccoli, kale, cabbage, spinach, and tomatoes.

Blood Handling

Blood samples were collected using sodium heprinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were centrifuged within 2 h of collection for 10 min at 1,200 × *g*. Immediately after centrifugation, serum was collected and three 1.5-mL alliquotes were taken and stored at −70°C.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokines

Peripheral blood mononuclear (PBM) cells were isolated from whole blood using a density gradient centrifugation on Ficoll-Paque (Pharmicia Biotech AB, Uppsala, Sweden) at 1,900 × *g* for 20 min. Cells were then collected and washed twice using sterile phosphate-buffered saline. Cell concentration was counted and adjusted to 1×10^6. Mononuclear cells were cultured in triplicate on a 96-well flat-bottomed culture plate (Falcon 3072, Lincoln Park, NJ) in RPMI-1640 culture medium with added 10% fetal calf serum. Cells were then stimulated with lipopolysaccharides (LPS) at 20 ng/mL or 20 μg/mL for 24 h for induction of interleukin-6 (IL-6) or tumor necrosis factor-α (TNF-α), respectively, or with phytohemaglutinin (PHA) at 10 μg/mL for 24 h for induction of IL-2 or 72 h for induction of interferon-γ (IFN-γ). Cells were incubated at 37°C in a 5% CO_2 incubator. After incubation, the plates were centrifuged for 10 minutes at 800 × *g*. Supernatant fluids were collected and stored at −70°C until analysis. The samples were then determined by sandwich ELISA [23] as we have described previously [24].

NK Cell Activity

NK cell activity was measured using a fluorescence release assay modified from the murine method of Poet et al. [26]. In brief, K562 cells-target cells were labeled with a fluorescent dye and incubated at different effector (PBMCs):target cell ratios (100:1, 50:1, and 25:1) in u-bottom tissue culture plates at 37°C in a humidified atmosphere of 5% CO_2 for 3 h. Epifluorescence of each well was determined and specific percent toxicity was calculated from fluorescence contained in target cells.

Determination of Carotenoids and Vitamin E in Serum

A total of 250 μL of ethanol (containing 0.1% butylated hydroxytoluene [BHT] antioxidant) were added to a 250-μL aliquot of serum to precipitate proteins. After vortexing, samples underwent two hexane extractions, evaporated under nitrogen, and then redissolved in mobile phase (solvent A). Then, 50 μL of extractant were injected directly into the high-performance liquid chromatography (HPLC) system. Carotenoids and α-tocopherol were separated by a 5-μm ultrasphere ODS column (4.6 × 250mm; Beckman Instruments, San Ramon, CA) and detected at the wavelengths of 452 nm and 300 nm by use of the method of Xu et al. [27]. The solvent system consisted of 95% acetonitrile (CAN)-tetrahydrofuran (THF) (85:15, v/v) with 250 ppm BHT and 0.05% triethylamine (TEA) and 5% 50 mM ammonium acetate in methanol with 0.05% TEA and was delivered at a flow rate of 2.5 mL/min. The retention times for lutein/zeaxanthin, β-cryptoxanthin, α-tocopherol, lycopene, α-carotene, and β-carotene were 1.94, 4.24, 4.83, 5.50, 9.45, and 10.13 min, respectively. The total run time for a single analysis of sample was 13 min. Analytic quantification was performed by the external standard method. Extinction coefficients were used to validate the final solution concentrations spectrophotometrically. Standard reference material 968b (fat-soluble vitamins and cholesterol in human serum) supplied by the National Institute of Standards and Technology (NIST, Gaithersberg, MD) was used for assigning values to in-house control materials.

Lymphocyte Subpopulation Measurement

Mononuclear cells were isolated as described above and adjusted to 2×10^6 cells/mL, 0.5 mL/tube, for subsequent lymphocyte surface marker determination as described previously [28]. Anti-human CD3 FITC/CD4 PE, CD3 FITCCD4 PE, or CD3 FITC/CD16 PE + CD 56 PE were obtained from Becton Dickinson (San Jose, CA). Samples were analyzed using a FacStar flow cytometer (Becton Dickinson, San Jose, CA) with the consort 40 program.

Statistics

Statistical significance was determined by using paired *t*-tests for the nutrient data (Microsoft Excel software, Copyright © Microsoft Corporation). Immune function data were analyzed separately for nonsmokers and smokers using one-way analysis of variance (ANOVA) (STATA). Bonferroni analysis was carried out to determine differences between means at baseline and day 40, baseline and day 80, and day 40 and day 80 (STATA). A p value of $<.05$ was considered statistically significant.

RESULTS

Subjects and Compliance

Subject characteristics are reported in Table 1. Subjects reported regular consumption of fruit and vegetable supplements. The supplements were well tolerated and improvements in bowel habits and overall feelings of well-being were noted. Pill counts were conducted each month, re-

Table 2. Effect of fruit and vegetable extracts on serum carotenoids and α-tocopherol levels

	Baseline	80 days	% Change	*p*
Lutein/zeaxanthin (μg/mL)	0.21 ± 0.084	0.28 ± 0.118	29.26	.0024
β-Cryptoxanthin (μg/mL)	0.08 ± 0.048	0.09 ± 0.047	12.20	NS
Lycopene (μg/mL)	0.30 ± 0.141	0.35 ± 0.141	15.53	.0424
α-Carotene (μg/mL)	0.07 ± 0.040	0.28 ± 0.130	308.08	.0001
β-Carotene (μg/mL)	0.33 ± 0.0311	0.88 ± 0.0554	165.24	.0001
α-Tocopherol (μg/mL)	22.56 ± 8.459	28.75 ± 9.815	24.43	.003

$N = 46$.
NS, not significant.

vealing that 99.99% of the fruit extract was consumed and 99.96% of the vegetable extract was consumed. No differences in immune function or serum antioxidants were observed for men and women (data not shown).

Serum Antioxidants

The average serum carotenoid and tocopherol concentrations are presented in Table 2. Lutein/zeaxanthin ($p < .05$), α-carotene ($p < .0001$), β-carotene ($p < .0001$), lycopene ($p < .05$), and α-tocopherol ($p < .005$) levels increased significantly after supplementation. No significant increase was seen for β-cryptoxanthin.

Mitogenesis and Lymphocyte Subpopulations

Spontaneous proliferation of PBM cells increased significantly ($p < .0001$) in nonsmokers (Fig. 1). The increase in spontaneous cell proliferation was seen when baseline was compared with day 40 ($p < .0001$) and day 80 ($p < .05$), and a slight decrease was seen when day 40 was compared with day 80 ($p < .01$). No significant differences were observed for smokers (data not shown).

No significant changes were noted in the amount of T helper cells ($CD3/CD4^+$), cytotoxic T cells ($CD3/CD8^+$), or NK cells ($CD3/CD16/CD56^+$) (data not shown).

NK Cell Cytotoxicity

NK cell cytotoxicity at an effector:target cell ratio of 100:1 ($p < .0001$), 50:1 ($p < .0001$), and 25:1 ($p < .005$) increased significantly in nonsmokers (Fig. 2). The increase in NK cell cytotoxicity was apparent when baseline was compared with day 80 and when day 40 was compared with day 80 at effector:target cell ratios of 100:1 and 50:1 ($p < .005$). At the effector:target cell ratio of 25:1, a significant increase was seen only when day 40 was compared with day 80 ($p < .005$). No significant differences were observed for smokers (data not shown).

Cytokines

IL-2 production in supernatant from PBM cells stimulated with PHA (10 μg/mL) increased significantly in smokers ($p < .05$) and nonsmokers ($p < .0001$) (Fig. 3). In smokers, significant increases in IL-2 were observed between baseline and day 80 and day 40 and day 80 ($p < .001$). In nonsmokers, significant increases were observed between

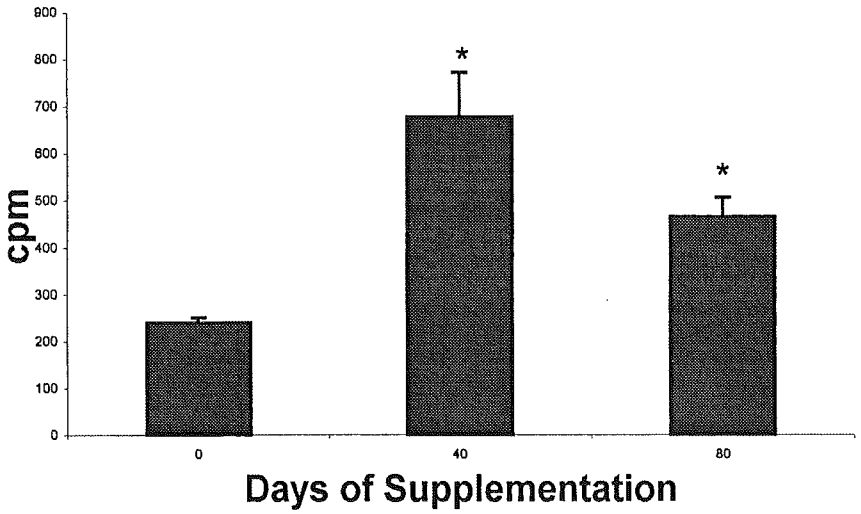

Figure 1. Effect of fruit and vegetable extracts on spontaneous proliferation of peripheral blood mononuclear (PBM) cells in elderly nonsmoking subjects. Each sample was done in triplicate and averaged. $N = 41$, 35, and 34 for baseline, day 40, and day 80, respectively. The values are mean ± SEM. *$p < .0001$.

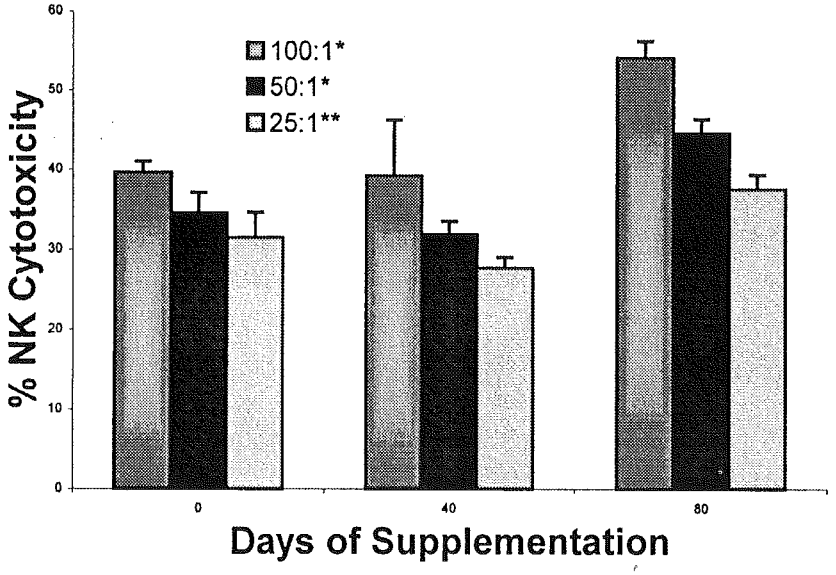

Figure 2. Effect of fruit and vegetable extracts on natural killer (NK) cell cytotoxicity of peripheral blood mononuclear (PBM) cells in elderly nonsmoking subjects. Each sample was done in triplicate and averaged. $N = 25$, 31, and 35 for baseline, day 40, and day 80, respectively. The values are mean ± SEM. $^*p < .0001$, $^{**}p < .005$.

baseline and day 40, baseline and day 80, and between day 40 and day 80 ($p < .0001$). In nonsmokers, IL-6 production in supernatant from PBM cells stimulated with LPS (20 ng/mL) decreased significantly ($p < .005$) between baseline and day 40; however, between day 40 and day 80 in this group, IL-6 levels increased significantly ($p < .05$). No statistically significant changes were observed when baseline values were compared with day 80 (data not shown). No significant changes were observed for either TNF-α or IFN-γ.

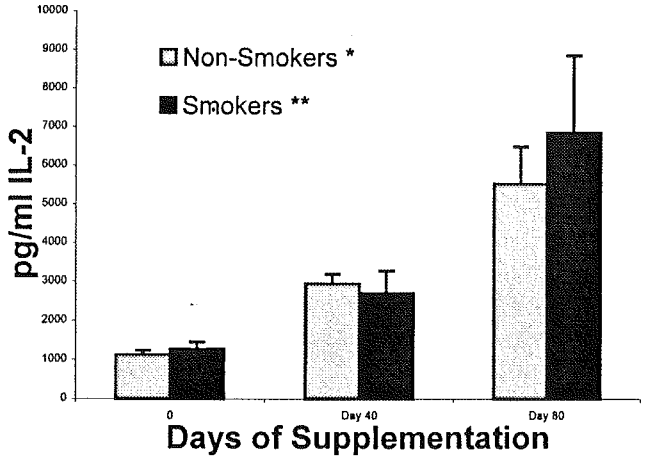

Figure 3. Effect of fruit and vegetable extracts on interleukin-2 (IL-2) production by peripheral blood mononuclear (PBM) cell stimulated with phytohemaglutinin (PHA) in nonsmoking and smoking elderly subjects. Each sample was done in triplicate and averaged. For nonsmokers, $N = 39$, 23, and 12 for baseline, day 40, and day 80, respectively. For smokers, $N = 8$, 6, and 4 for baseline, day 40, and day 80, respectively. The values are mean ± SEM. $^*p < .0001$; $^{**}p < .05$.

DISCUSSION

The present study is the first to describe how fruit and vegetable extracts can be used to significantly improve immune function in the elderly. The supplements were processed by a unique method that extracts the constituents of fruits and vegetables so their bioavailability is enhanced. We believe that our data underscore the importance of supplementing diets in the elderly when the optimum method, high consumption of many different fruits and vegetables, cannot be guaranteed.

Aging results in a dysregulation of immune function. Cell-mediated immunity tends to become suppressed whereas humoral immunity is elevated. The decline in cell-mediated immunity, IL-2, and T-cell function is thought to be a result of thymus involution. B cells, on the other hand, are generated throughout life in humans [29]. The decline in T-cell function in combination with the continued production of B cells leads to a dysregulated immune response. Increased humoral immunity results in aberrant antibody production, which can be devastating, as indicated by the prevalence of autoimmune diseases in the elderly. Impaired cell-mediated immunity is associated with an inability to destroy viruses and cancer cells. Interestingly, as immune function declines, the incidence of CVD and cancer rises. Finding ways to reduce risk for these diseases as well as improve immune function would be extremely useful. Increasing consumption of fruits and vegetables is the ideal way to lower risk. Many seniors, due to the high cost of vegetables and fruits, and reduced calorie needs, and because of problems in digesting some plant materials, have lower intake and, thus, lower exposure to the small molecular weight materials in plants. Because few older individuals consume the optimal amounts of fruits and vegetables, they need methods to increase intake of

plant constituents. Thus, a dietary supplement has potential importance. At first, studies in animals [11–14] given individual supplemental antioxidant vitamins were encouraging; however, the evidence in humans [15,16] is not as clear. T- and B-cell proliferation in old rat splenocytes, along with IL-2 levels, have been shown to increase in response to dietary vitamin E [11]. In old mice fed high vitamin E diets, IL-2 was also shown to increase [12,13]. However, in a study in which elderly humans were supplemented with 100 mg of vitamin E, no changes in PBM cell proliferation were observed [15]. Similarly, β-carotene increased the in vitro stimulation of mouse splenocytes [14], but failed to increase PBM cell proliferation in humans [16]. Additionally, IL-2 production was also unchanged in elderly subjects supplemented with β-carotene [16]. Antioxidants such as β-carotene and vitamin E may need to work in tandem with other nutritive and/or non-nutritive compounds to exert in vivo effects (as when a mixture of vitamins and trace elements were supplemented in a group of healthy elderly subjects, it prevented further suppression of cell-mediated immunity [30]). This finding, therefore, suggests that nutrients are working together to exert their functions. Because not all of the beneficial agents present in fruits and vegetables have been identified, at best we can only prevent further suppression when specific nutrients are given in combination [30]. We propose that the improvements we observed in immune function are due to the action of nutritive and non-nutritive agents present in the extracts, as our findings show improvements in IL-2 levels, NK cytotoxicity, and LPS and spontaneous PBM cell proliferation.

Decreases in IL-2 production in the elderly are attributed to decreases in cell-mediated immunity. Our data demonstrate that fruit and vegetable extracts can improve IL-2 levels markedly, in both smokers and nonsmokers, as we observed twofold increases after only 40 days of supplement use. A benzene derivative found in cigarette tar, *p*-benzoquinone (p-BQ), and nicotine, were both found to inhibit IL-2 production in PBM cells and locally suppress lung cell-mediated immunity [31,32]. The increase in IL-2 we observed may therefore have added benefits for elderly smokers. Because smokers are at such high risk for developing lung cancer, it is essential to find ways to reduce their risk. However, recent evidence [8,9] suggests that antioxidant supplementation increases the risk of developing lung cancer in smokers. The importance of other antioxidants or substances in fruits and vegetables, and their synergistic effects came to be appreciated. We are now able to demonstrate that fruit and vegetable extracts, containing both nutritive and non-nutritive compounds, can be of use to the smoking and nonsmoking elderly populations, as indicated by enhanced production of IL-2.

Another important aspect of our findings is that twofold increases in IL-2 and spontaneous cell proliferation occur after only 40 days of supplementation . The age-associated decline in spontaneous mitogenesis has been attributed to a decline in IL-2 [33]. Additionally, IL-2 antagonists increase with age, thereby decreasing IL-2's effectiveness [34]. Because we observed increases in LPS and spontaneous cell proliferation and IL-2 levels, perhaps some components in the fruit and vegetable extracts work to upregulate IL-2 production. Increased IL-2 could then modulate other aspects of immune function and improve cell-mediated immunity.

IL-6, an inflammatory cytokine, also involved in activation, growth, and differentiation of T cells [35], is known to be higher in the elderly [36]. Our data show significant decreases in IL-6 levels between baseline and day 40; however, these levels increase significantly from day 40 to day 80. Overall, when baseline values were compared with day 80, no significant changes occurred (data not shown). It is therefore unclear whether fruit and vegetable extracts have effects on IL-6 levels. Our finding of improved NK cell cytotoxicity, after 80 days of supplement use, at each of the effector:target cell ratio is also of extreme importance. Because the incidence of cancer increases with age, finding ways to reduce risk is an effective approach to reducing cancer mortality rates. NK cells are capable of destroying cancer cells and have been shown to have lower function in older mice [37], older humans [38], and patients with oral cavity cancer [39]. We observed that the number of NK cells did not change, as indicated by our flow cytometry data (data not shown), but that the cells became more cytotoxic. This finding is in agreement with previous data we observed in mice supplemented with vitamin E [12]. Others observed increased NK activity in humans supplemented with β-carotene [40] which was not due to increases in the number of NK cells [16,41,42]. The mechanism by which NK activity is increased may be mediated by IL-2 levels. IL-2 is reduced in the elderly, and also is capable of enhancing NK cytotoxicity [43]. Because, as we have also shown, IL-2 production by PBM cells increased after supplementation, the increase in NK cytotoxicity may be occurring secondary to the enhanced IL-2 production.

We observed a highly significant increase in serum carotenoids and α-tocopherol levels, indicating that the supplements were well tolerated and compliance was high. The pretreatment values for these nutrients were similar to those found by others researchers [21,44,45], which illustrates that our sample was representative of most elderly populations. In other subjects given diets high in fruits and vegetables, similar increases in these nutrients after two 15-day periods on the diet were observed [45]. This finding demonstrates that fruit and vegetable *extracts* contain many of the same components that fruits and vegetables themselves have, and that the antioxidants we measured are digested and absorbed in a similar fashion. This finding is extremely relevant in view of the fact that few individuals consume the recommended number of servings of fruits and vegetables [18], and that until now, an acceptable alternative has not been available.

The improved immune function may be due to reduced DNA splits or damage [46]. Using cells from our subjects, Smith et al. [46] showed recently that the subjects' lym-

phocytes had more intact DNA, and thus more functional potential after consumption of the fruit and vegetable extracts for 80 days. Their measurements expand further the benefits found in the small molecular weight materials in plants, whether obtained by consumption or in commercial extracts.

In conclusion, fruit and vegetable extracts are a safe and well-tolerated supplement to dietary fruits and vegetables. They are also beneficial at improving immune function in the elderly who have increased risk for developing infections, cancer, and heart disease. The supplements were also useful in smokers, as indicated by the increase in IL-2. Ultimately, fruit and vegetable extracts could be a useful tool to improve immune function in elderly. However, the presence of fiber with its multiple benefits continues to support consumption of a broad range of fruits and vegetables as the optimum choice to obtain the beneficial small molecular weight materials that were immunorestorative in this study.

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