

Effects of β -Carotene and α -Tocopherol on Bleomycin-induced Chromosomal Damage¹

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Abstract

The number of bleomycin-induced chromosomal breaks in cultured peripheral blood lymphocytes has been proposed as a measure of the sensitivity of an individual to carcinogens. Although "mutagen sensitivity" (clastogenicity) may be a useful biomarker for the identification of individuals at high risk for DNA damage, there is some uncertainty whether the results of this assay can be modified by environmental factors, such as diet. We designed an intervention study to determine whether micronutrient supplementation with β -carotene and α -tocopherol influenced the mutagenicity score among 22 healthy volunteers. This intervention study followed a double-blind, randomized, cross-over design. Chromatid breaks ranged from 0.30 to 2.30 per cell and were uncorrelated with plasma β -carotene ($r = -0.07$; $P = 0.50$) and α -tocopherol ($r = -0.01$; $P = 0.92$) levels, after accounting for the time of the measurement. The average number of breaks per cell was similar (P for difference in means = 0.90) among subjects during periods of vitamin supplementation (mean = 0.87 breaks per cell) and placebo (mean = 0.86 breaks per cell), averaged over groups and after adjustment for baseline breaks. Substantial within-person variation may indicate some imprecision in the mutagen sensitivity assessment. Our results suggest that mutagen sensitivity is not affected by plasma levels of β -carotene or α -tocopherol. Although mutagen sensitivity does not appear to be modified by changes in plasma levels of two common antioxidant vitamins, it may be useful for the identification of high-risk individuals for participation in large intervention studies with cancer outcomes.

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Introduction

The identification of heritable factors that modify human sensitivity to genetic damage would improve cancer risk assessment (1). Hsu (2) has suggested that if chromosome fragility is an indicator of genetic susceptibility to cancer, then individuals whose cultured peripheral blood lymphocytes are more sensitive to bleomycin-induced chromosomal breaks may be at greater risk of developing a variety of cancers. Therefore, the number of bleomycin-induced chromatid breaks per cell may be a measure of "mutagen sensitivity" or susceptibility to environmental carcinogens (3). Indeed, this measure of mutagen sensitivity has been associated with cancers of the lung and upper aerodigestive tract (4, 5) and appears predictive of the development of second primary tumors in patients who are cured of head and neck cancer (6, 7).

The use of antioxidant micronutrients and dietary modification to inhibit the promotional phase of carcinogenesis is the central focus of several ongoing chemoprevention and dietary intervention studies. There is presently great interest in biomarkers that can be used to identify target populations that might benefit most from large population interventions (1). Although it is assumed that mutagen sensitivity is a genetically transmitted phenotype, results from an earlier study by our group showed that the number of bleomycin-induced breaks per cell was correlated with plasma levels of several antioxidants (8). Others have shown a reduction in the number of bleomycin-induced chromatid breaks with the introduction of 13-*cis*-retinoic acid (9), α -tocopherol, *n*-acetyl-L-cysteine (10) to lymphoid cell lines *in vitro*, or with vitamin C supplementation *in vivo* (11). We designed a small, randomized trial to examine the possibility that dietary supplementation with β -carotene and α -tocopherol reduces mutagen sensitivity in a healthy, non-smoking population of men and women.

Materials and Methods

This intervention study followed a double-blind, randomized, cross-over design. Volunteers included healthy persons with no medical conditions that would be affected by vitamin supplementation or blood specimen collection. Pregnant or nursing mothers and persons undergoing cancer treatment or treatment for other medical conditions that might have affected participation in the study were also excluded. All participants were nonsmokers and were not taking any vitamin supplements for a minimum of 6 weeks before the start of the study. Most participants were employees of the Cancer Research Center of Hawaii. This study was approved by the Committee on Human Subjects at the University of Hawaii and the Clinical Protocol Review Committee at the Cancer Research Center of Hawaii.

Twenty-two healthy volunteers (16 women and 6 men) agreed to participate and signed informed consent. At entry, volunteers were randomized evenly into two groups. The first group (group 1) took 15 mg of β -carotene and 400 IU of α -tocopherol twice daily (with breakfast and dinner) for a

period of 6 weeks after the baseline blood sample was obtained. The second group (group 2) took a placebo twice daily for the 6 weeks after the initial blood draw. Neither the subjects nor the investigators knew the group assignments until after study completion. A 6-week supply of pills were given to each of the volunteers at the start of the study. Subjects were allowed to follow their regular diet during the entire study period and were asked not to take any vitamin/mineral supplements other than those provided by the study.

Week 6 through week 12 was used as a wash-out period during which no intervention was conducted. At week 13, the cross-over occurred and volunteers taking placebo during the first stage of the trial were given vitamin supplements, and volunteers initially in the intervention group were placed on placebo. The trial ended after week 18.

Volunteers had 10 cc of blood collected in heparin-containing Vacutainer tubes after an overnight fast four times during the study period: at baseline, at the end of week 6, at the end of week 12, and at the end of week 18. After collection, whole blood was packaged in an insulated mailing kit and sent by express courier to the University of Texas M. D. Anderson Cancer Center for mutagen sensitivity analysis. The remaining blood was stored immediately on ice and protected from light before and during centrifugation and sample processing. Plasma was obtained by centrifugation of the blood at $1400 \times g$ for 10 min. Plasma samples were stored at -70°C and analyzed for selected antioxidants at the end of the study.

Mutagen Sensitivity Assay. The mutagen sensitivity assay was performed as described in detail by Cherry and Hsu (12). Briefly, heparinized (sodium heparin) whole blood (1 ml) was added to a culture flask containing RPMI 1640 (9 ml) supplemented with penicillin, streptomycin, 15% FCS, and 0.1 ml of reconstituted phytohemagglutinin and incubated for 72 h at 37°C (5% CO_2). During the last 5 h of incubation, the lymphocytes were exposed to bleomycin at a final concentration of 0.03 unit/ml and, during the last hour of incubation, Colcemid (final concentration, 0.04 $\mu\text{g}/\text{ml}$) was added to the culture medium to arrest the cells in metaphase before harvesting. Harvested lymphocytes were exposed to 0.06 M potassium chloride at room temperature for 20 min. After the hypotonic treatment, 10% freshly prepared Carnoy fixative (3:1 methanol: acetic acid) was added, mixed thoroughly, and centrifuged at $800 \times g$ for 5 min. The cells were then fixed with Carnoy's mixture for a minimum of 10 min. Three washings with Carnoy's mixture were performed, and chromosome preparations were made using wet slides. Air-dried slides were stained with 4% Giemsa in 0.01 M phosphate buffer. The number of chromatid breaks per cell was quantitated by analyzing 50 lymphocytic metaphases.

Plasma Micronutrient Assay. Plasma β -carotene and α -tocopherol were analyzed by high pressure liquid chromatography with diode-array detection (13). Ethanol containing bis-hydroxy-toluene as antioxidant and three internal standards were added to plasma to precipitate proteins. Subsequently, the liberated lipophilic micronutrients were extracted by repeated (three times) hexane extraction. The combined hexane layers were dried under nitrogen and dissolved in the high pressure liquid chromatography mobile phase consisting of methanol: dichloromethane:acetonitrile (65:25:10), bis-hydroxy-toluene (0.025%) as antioxidant, and aqueous bis-tris-propane (2 ml/l of 0.5 M, pH 7.0) as buffer to prevent on-column degradation. β -carotene and α -tocopherol were separated on a Spherex 5- μm C_{18} column (250×4.6 mm; Phenomenex, Torrance, CA) and monitored by a dual multiple wavelength diode-array

detector at each individual compound's absorption maximum. Micronutrient levels were determined using peak areas and calibration curves of authentic standards.

The accuracy and precision of the micronutrient assay was assessed in a quality assurance program for lipid-soluble micronutrients organized by the National Institute of Standards and Technology (Gaithersburg, MD). During the period of this study, 11 plasma samples were included in this round-robin exercise. For β -carotene and α -tocopherol, our mean deviation from the accepted value was calculated to be 6.5 and 3.3%, respectively, whereas the coefficient of variation for within-assay repeatability was found to be 2.8 and 2.4%, respectively. Blinded duplicate analysis of the 11 samples from this study resulted in coefficients of variation of 4.8 and 1.9% for β -carotene and α -tocopherol, respectively.

Random analysis of the supplements confirmed the dosage, with deviations of up to 20%. β -Carotene placebos were found to contain negligible β -carotene levels (<0.08 ng). α -Tocopherol placebos were found to contain 0.04, 0.21, and 0.08 mg of α -, γ -, and δ -tocopherol, respectively, probably because plant oil (soy oil) was used as the matrix. The presence of α -tocopherol in the placebos was also negligible, considering that the amount detected was equivalent to 0.01% of the amount in the supplements.

Statistical Analysis. We first examined the distribution of the chromatid breaks per cell and the plasma micronutrient levels. The distribution of chromatid breaks was approximately symmetrical, but the distributions of β -carotene and α -tocopherol were skewed to the right, and log-transformation was performed to meet the analytic assumptions. Levels of β -carotene and α -tocopherol were plotted against the day at which blood was drawn to evaluate study compliance. Partial Pearson's correlation coefficients were used to determine the linear relationship between variables while adjusting for covariates. We used the mixed model (14) to assess the effect of vitamin supplementation on chromatid breaks. In this model, person was treated as a random effect, each contributing four observations, and all other factors were treated as fixed effects. The dependent variable was chromatid breaks per cell, and the primary exposure variables included vitamin supplementation (β -carotene/ α -tocopherol *versus* placebo), group (intervention first or group 1 *versus* placebo first or group 2), and a carry-over effect (interaction between supplementation and group). Baseline chromatid breaks per cell (at week 0) served as an adjustment variable. Because there was no vitamin supplementation effect, we combined the groups to look at the within- and between-subject variation in chromatid breaks over time in the model above, with only the random effect of person included.

Results

The mean age of volunteers was 43 years for group 1 and 40 years for group 2. Baseline plasma levels of β -carotene and α -tocopherol were similar between group 1 (intervention/placebo) and group 2 (placebo/intervention) (Figs. 1 and 2). Subsequent blood measurements suggest compliance with the intervention scheme. As expected, group 1 experienced a steady rise in plasma levels of β -carotene and α -tocopherol, followed by a decline after week 6. Plasma levels of β -carotene and α -tocopherol were constant among subjects in group 2 through week 12. A substantial increase in plasma micronutrient levels was detected in week 18 after 6 weeks of supplementation.

Plasma levels of β -carotene and α -tocopherol were strongly correlated ($r = 0.62$, $P = 0.0001$). However, plots of chromatid breaks against logged plasma levels of β -carotene

Fig. 1. Mean plasma β -carotene levels for the two study groups. Group 1: vitamin supplement followed by placebo; group 2: placebo followed by vitamin supplement.

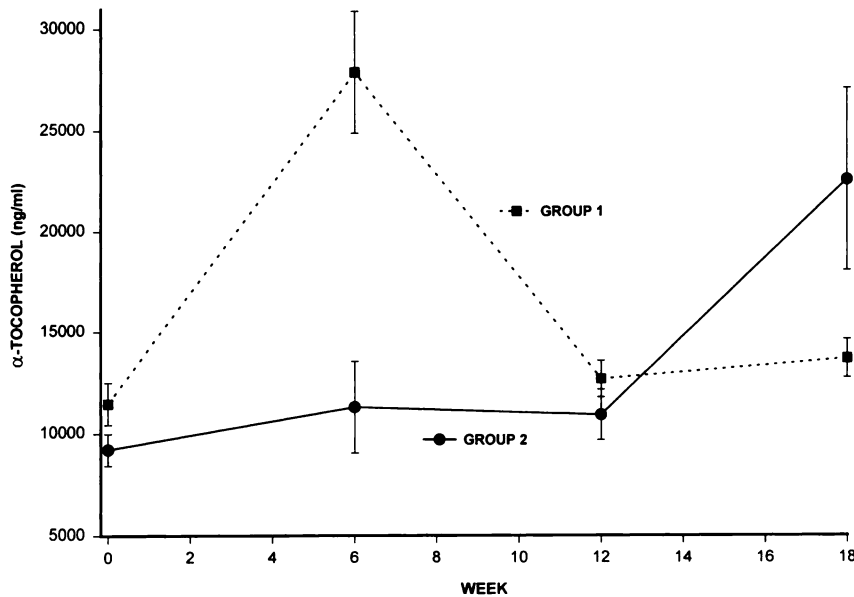
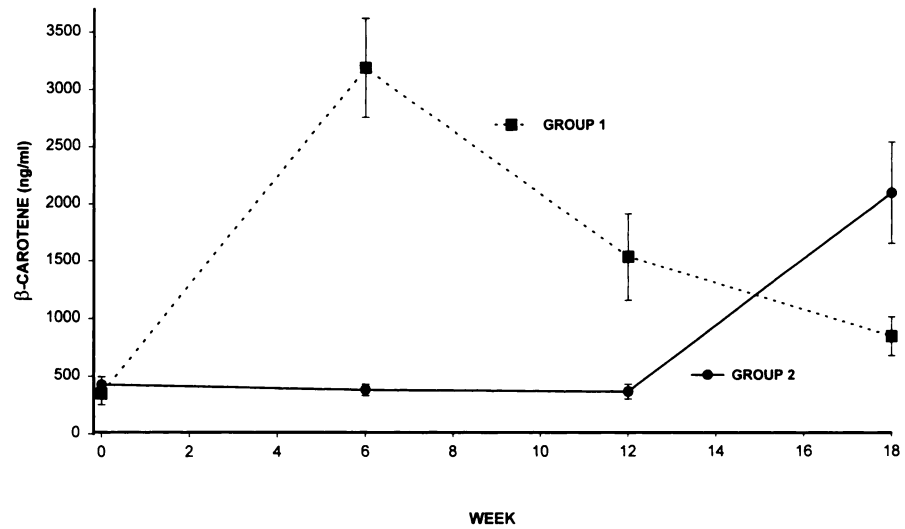


Fig. 2. Mean plasma α -tocopherol levels for the two study groups. Group 1: vitamin supplement followed by placebo; group 2: placebo followed by vitamin supplement.

and α -tocopherol suggested little correlation between mutagen sensitivity and micronutrient levels (data not shown). Chromatid breaks were poorly correlated with plasma β -carotene levels ($r = -0.07$; $P = 0.50$) and plasma α -tocopherol levels ($r = -0.01$; $P = 0.92$), partialing out the effect of the time of the measurement.

Fig. 3 shows the unadjusted mean breaks per cell levels for groups 1 and 2 during the 18-week study period. Chromatid breaks per cell ranged from 0.30 to 2.30 at baseline. We found no effect of vitamin supplementation or group on the mean chromatid break level, before (data not shown) or after adjustment for baseline break levels (Table 1). The covariate-adjusted mean breaks per cell was 0.86 for the placebo group and 0.87 for the vitamin supplementation group. The difference of 0.01 chromatid breaks per cell between the two groups was not significant ($P = 0.90$). However, there was a significant ($P = 0.01$) 23% difference in the mean baseline breaks per cell

between subjects in group 1 (mean = 1.14, SD = 0.59) and subjects in group 2 (mean = 0.88, SD = 0.34).

The within-subject variance component of breaks per cell (0.12) was considerably greater than the between-subject variance component (0.05). We also computed these variance components among subjects receiving placebo or no intervention (group 1 subjects at weeks 6, 12, and 18 and group 2 subjects at weeks 0, 6, and 12). Again, the within-subject variance component was greater than the between-subject variance component.

Discussion

Results of this small, randomized, cross-over intervention trial among healthy nonsmokers failed to show that supplementation with β -carotene/ α -tocopherol leads to reduced bleomycin-induced chromosomal damage. In this study, mutagen sensitivity

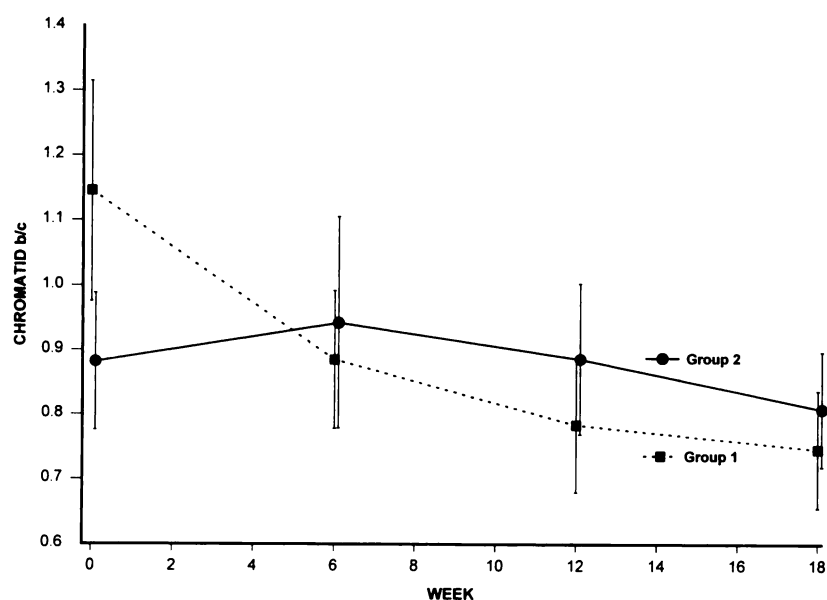


Fig. 3. Mean chromatid breaks per cell for the two study groups. Group 1: vitamin supplement followed by placebo; group 2: placebo followed by vitamin supplement.

Table 1 Results of mixed-model analysis of variance of chromatid breaks per cell by vitamin, group, vitamin-by-group, and baseline chromatid breaks

Comparison ^a	β	SE	Two-sided P
Vitamin	-0.095	0.148	0.90
Group	-0.248	0.145	0.18
Vitamin by group	0.215	0.201	0.29
Baseline	0.269	0.105	0.01

^a Vitamin, vitamin supplement versus placebo; Group, vitamin supplement followed by placebo versus placebo followed by vitamin supplement; Baseline, chromatid breaks per cell at baseline (week 0).

level, or the sensitivity to bleomycin-induced chromosomal breakage, was not modified by either of these antioxidants. As such, the mutagen sensitivity assay does not appear to be an appropriate intermediate marker for intervention effects in chemoprevention studies using these antioxidants.

A number of *in vitro* studies have been conducted to examine the effects of antioxidants on genotoxicity and to develop an *in vitro* model of their protection against genetic damage. Weitberg *et al.* (15) found that α -tocopherol significantly reduced phagocyte-induced sister chromatid exchanges. Stich and Dunn (16) reported that β -carotene inhibited micronuclei formation and chromatid aberration *in vitro* of some carcinogens but not others. Trizna *et al.* (9, 10) have performed studies on bleomycin sensitivity *in vitro* using several chemopreventive agents, including α -tocopherol, 13-*cis*-retinoic acid, *N*-acetylcysteine, and ascorbic acid. Although excessive amounts of these agents in the medium reduced the chromatid breakage frequency induced by bleomycin, the antioxidants needed to be added to the medium at least 2 h before the introduction of the bleomycin for protection to occur.

Only a few investigators have examined the *in vivo* association of dietary or plasma antioxidant concentrations and mutagen sensitivity in healthy populations. In a small investigation among 25 healthy, nonsmoking volunteers, Kucuk *et al.* (8) found significant inverse correlations between mutagen sensitivity and plasma levels of α -carotene, β -carotene, total

carotenoids, retinol, and vitamin C. Pohl and Reidy (11) reported a significant decrease from baseline in the number of bleomycin-induced chromatid breaks after 4 weeks of vitamin C supplementation (100 mg/day for the first 2 weeks and 1000 mg/day for the following 2 weeks) among eight healthy volunteers.

This the first intervention trial, to our knowledge, in which the effects of two potential chemopreventive agents, β -carotene and α -tocopherol, on chromosome fragility have been examined. Our findings are consistent with those of Cloos *et al.* (17, 18) and Spitz *et al.* (7, 19), who found no association of a broad range of environmental factors with mutagen sensitivity scores among cancer patients. Cloos *et al.* (18) compared mutagen sensitivity levels among 19 head and neck cancer patients who had received 600 mg/day of *N*-acetylcysteine with 14 patients who did not receive supplementation. In contrast to the findings of Trizna *et al.* (10), there was no evidence that *N*-acetylcysteine influenced the mutagen sensitivity levels among these cancer patients. In a recently published study of 612 patients with squamous cell carcinoma of the upper aerodigestive tract, Spitz *et al.* (19) showed that the dietary consumption of various carotenoids and vitamin C were not correlated with mutagen sensitivity. We concur with these investigators that the number of chromatid breaks per cell inducible by bleomycin *in vitro* is likely a constitutional factor and unrelated to environmental exposures.

In this study, within-subject variance was considerably greater than between-subject variance for the mutagenicity assay, suggesting some imprecision or instability in the assay, at least in the range of values observed in our healthy study population. The within-person variance component reflects both the laboratory variability of the mutagen sensitivity assay, as well as the presumably random temporal variability in breaks per cell over the four time periods. An analysis of the reliability of the mutagen sensitivity assay has shown that scoring 50 metaphases per sample yields acceptable accuracy (20). Although large variability would limit the usefulness of this susceptibility marker among studies in which mean differences between groups are small, the mutagen sensitivity assay may be useful to identify individuals at high risk of cancer for partic-

ipation in intervention studies that are large enough to minimize the importance of the intraindividual variation. Future studies should examine both the laboratory and intraindividual variability of the assay results in persons with a wide range of mutagen sensitivity levels.

Absence of information on the adequacy of the length of the wash-out after administration of the vitamin supplements and the small sample size are limitations of this study. In our study, a difference in means of 0.05 could be detected with a power of 0.80 and α of 0.05 (two-sided). The power was inadequate to detect a difference in means as small as that observed (0.01), but this difference is probably not clinically meaningful. Because our participants were all healthy with low mutagen sensitivity scores, we cannot exclude the possibility that vitamin supplements may be effective at reducing the number of chromatid breaks among individuals with higher baseline readings. If this is true, the continued reduction in breaks per cell for group 1 after vitamin supplementation could have resulted from a residual effect of supplements on mutagen sensitivity. We had no means of testing this possibility within the design constraints of the trial.

In addition to complexing with cellular DNA, antioxidants exist in the nucleus and cytoplasm at equilibrium with the microenvironment. When blood was cultured for the mutagen sensitivity assay, we added 1 ml of blood to 9 ml of medium, thus diluting the antioxidant concentration 10-fold. It is possible that *in vitro* dilution of the antioxidant concentrations achieved in plasma may have attenuated an effect of β -carotene/ α -tocopherol, especially if antioxidants have an immediate impact on chromatid breaks. Future studies might consider adding antioxidant to the medium up to its original concentration within the 2 h before introduction of bleomycin. This addition would maintain *in vivo* intracellular levels of α -tocopherol and β -carotene during the performance of the *in vitro* assay, without causing the protective effect of added antioxidants found by Trizna *et al.* (9, 10).

If the number of chromatid breaks is, indeed, a stable measure of mutagen sensitivity and if, in turn, mutagen sensitivity is a biomarker of genetic susceptibility to cancer after exposure to genotoxic agents, then mutagen-sensitive persons may be potential candidates for chemoprevention or dietary intervention trials that seek to reduce the risk of cancer. In the future, it may be practical to use the bleomycin assay as a way to identify high-risk individuals for intervention studies.

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