[CANCER RESEARCH 56, 1291–1295, March 15, 1996]

Antioxidant Supplementation Decreases Oxidative DNA Damage in Human Lymphocytes[1]

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ABSTRACT

The association between high intake of fruit and vegetables and low incidence of certain cancers is well established. Dietary antioxidants present in these foods are thought to decrease free radical attack on DNA and hence to protect against mutations that cause cancer, but this causal mechanism remains conjectural. We have adopted a molecular epidemiological approach to this question, based on a modified alkaline single-cell gel electrophoresis assay ("comet assay") which specifically detects oxidation of pyrimidines in the DNA of human lymphocytes. In a survey of men 50–59 years of age living in the northeast of Scotland, smokers initially showed significantly more base damage than nonsmokers. Correlations between oxidative base damage and plasma concentrations of various antioxidants were generally negative but not statistically significant.

Supplementation of the diet for 20 weeks with vitamin C (100 mg/day), vitamin E (280 mg/day), and β-carotene (25 mg/day) resulted in a highly significant ($P < 0.002$) decrease in endogenous oxidative base damage in the lymphocyte DNA of both smokers and nonsmokers. In addition, lymphocytes of antioxidant-supplemented subjects showed an increased resistance to oxidative damage when challenged *in vitro* with H_2O_2.

These findings strongly support the hypothesis that fruit and vegetables exert a cancer-protective effect via a decrease in oxidative damage to DNA.

INTRODUCTION

Endogenous damage to DNA, caused by oxygen-free radicals liberated during normal respiration, may be significant in the aetiology of cancer (1). DNA damage can account for the genetic changes that occur at the different stages in the progression from anaplastic growth to metastasis (2), and therefore dietary factors that reduce the impact of free radical attack are likely to protect against cancer. Epidemiological studies indicate that a diet rich in fruit and vegetables is indeed associated with lower incidence of various forms of cancer (3–6). This protection is commonly attributed to the dietary content of antioxidants such as vitamin C, vitamin E, and various carotenoids (7–10). Few intervention trials have been carried out to test this hypothesis, since large numbers of volunteers are needed, and several years must elapse before sufficient cases of disease have arisen. The recent Linxian trial demonstrated a significant effect of a combination of vitamin E, β-carotene, and selenium on mortality, particularly from esophageal cancer (11). At the same time, however, conflicting results have been reported, notably in the recent Finnish study (12), in which smokers were given supplements of vitamin E, β-carotene, or both for up to 8 years. Unexpectedly, the incidence of lung cancer was significantly higher in those subjects receiving β-carotene compared with the placebo group. Inevitably, the validity of the study design and its analysis has been scrutinized, and this illustrates the difficulty in designing universally acceptable epidemiological trials. Some unpredicted biological effects of large doses of β-carotene have to be considered, such as a possible inhibition of the absorption of other, perhaps more effective antioxidant carotenoids from food. On supplementation with β-carotene, both increases and decreases in plasma levels of other carotenoids have been reported (13, 14). Clearly, there is a need for more information on the biochemistry of antioxidant protection in the whole organism before further large scale and expensive epidemiological studies are undertaken.

Molecular epidemiology offers an alternative, more focused, and economical approach. Biomarkers of DNA damage or mutation are measured as indicators of carcinogenic risk. A regimen of dietary supplementation with antioxidant micronutrients, applied in a controlled way to a carefully selected representative sample of the population, can be expected to yield definitive answers to questions concerning the efficacy of antioxidant protection.

We have carried out a double-blind supplementation study on small, well-defined groups of smokers and nonsmokers, half of each group receiving a daily antioxidant supplement, and the other half a placebo, for up to 40 weeks. At intervals, we measured antioxidant concentrations in plasma and biomarkers of genetic change. The "comet assay," or SCGE,[3] was used to estimate DNA strand breaks and oxidized bases. We report here the substantial protective effect provided by antioxidant supplementation, as indicated by *(a)* a decrease in endogenous oxidative damage to bases in DNA and *(b)* an increase in the resistance of lymphocytes to oxidative damage inflicted *in vitro*.

MATERIALS AND METHODS

Materials. Histopaque 1077 and RPMI 1640 medium without phenol red and bovine serum albumin fraction V were obtained from Sigma (Poole, England). Ultrapure low melting point agarose and standard melting point agarose (both electrophoresis grade) were supplied by Gibco Life Technologies (Paisley, Scotland). Fully frosted glass microscope slides were obtained from Richardson Supply Co. (London, England), and 4′,6-diamidine-2-phenylindole dihydrochloride was obtained from Boehringer Mannheim (Lewes, England). FCS was obtained from Globepharm Ltd. (Esher, England). Dr. R. Cunningham (Department of Biological Sciences, State University of New York, Albany, NY) generously supplied the *Escherichia coli* strain overproducing endonuclease III. Capsules containing vitamin C, vitamin E, and β-carotene in hydrogenated coconut oil and placebo capsules containing just oil were a gift from BASF (Ludwigshafen, Germany).

Selection of Subjects and Blood Sampling. Our volunteers were normal healthy males between the age of 50 and 59 years. Individuals on drug therapy or health supplements or with a history of heart disease were excluded. About 50 smokers and 50 nonsmokers were recruited. Nonsmokers were defined as those who reported never having smoked; ex-smokers were not used in this study. They were assigned at random to the supplement or placebo group. The supplements of vitamin C (100 mg), vitamin E (α-tocopherol, 280 mg), and β-carotene (25 mg) or placebo were taken daily, and blood samples were collected by venipuncture (after overnight fasting) at 0, 5, 10, 20, and, in some cases, 40 weeks to determine DNA damage in lymphocytes using SCGE; plasma antioxidants including vitamins C and E and β-carotene were also measured.

The study was approved by the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen.

DNA Damage Measured by SCGE. Thirty μl whole blood were mixed with 1 ml RPMI 1640 medium supplemented with 10% (v/v) FCS and kept on

Received 9/25/95; accepted 1/12/95.

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[1] Supported by the Ministry of Agriculture, Fisheries, and Food and the Scottish Office Agriculture and Fisheries Department.

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[3] The abbreviation used is: SCGE, single-cell gel electrophoresis.

Table 1 *Plasma antioxidant levels: effects of supplementation for 20 weeks*[a]

	α-Tocopherol/cholesterol (μM/mM)		β-Carotene (μg/ml)		Ascorbate (μM)	
	Week 0	Week 20	Week 0	Week 20	Week 0	Week 20
Smokers, placebo	4.92 ± 0.28 (n=19)	4.76 ± 0.18 (n=18)	0.29 ± 0.03 (n=19)	0.24 ± 0.02 (n=18)	26.4 ± 5.0 (n=21)	26.2 ± 3.6 (n=19)
Smokers, supplement	4.20 ± 0.15 (n=16)	6.19 ± 0.33 (n=16)	0.21 ± 0.02 (n=11)	1.26 ± 0.16 (n=16)	26.4 ± 5.4 (n=18)	54.6 ± 3.1 (n=18)
Nonsmokers, placebo	4.79 ± 0.18 (n=20)	5.54 ± 0.18 (n=23)	0.31 ± 0.03 (n=21)	0.33 ± 0.03 (n=23)	38.2 ± 3.2 (n=25)	46.3 ± 2.3 (n=25)
Nonsmokers, supplement	4.91 ± 0.15 (n=25)	6.59 ± 0.24 (n=23)	0.33 ± 0.02 (n=24)	1.93 ± 0.17 (n=23)	37.2 ± 3.5 (n=30)	52.1 ± 2.7 (n=28)

[a] Mean values ± SE. Differences in individual antioxidant concentrations between weeks 0 and 20 were significant in all cases ($P < 0.001$) for supplemented subjects, both smokers and nonsmokers.

ice for 30 min. Lymphocytes were isolated after centrifuging over 100 μl Histopaque 1077 at 200 × *g* for 3 min at 4°C, washed once in PBS (pH 7.4), and centrifuged again. The cells recovered were confirmed to be 95% mononuclear cells (data not shown).

DNA damage was induced *in vitro* by exposing lymphocytes to H_2O_2 in PBS for 5 min on ice. Treatment on ice minimizes the possibility of cellular DNA repair.

DNA breaks and oxidized pyrimidine bases were measured using the alkaline comet assay as described previously (15–17). In brief, lymphocytes (from 30 μl blood, with or without H_2O_2 treatment) were suspended in 80 μl 1% low melting point agarose in PBS at 37°C and pipetted immediately onto a frosted glass microscope slide precoated with 80 μl 1% high melting point agarose, similarly prepared in PBS. The agarose was allowed to set by incubating at 4°C for 10 min, and the slides were then immersed in lysis solution (1% Triton X-100, 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, pH 10.0) at 4°C for 1 h to remove cellular membranes, proteins, and so forth. Slides were then placed in a single row in a 260-mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA at 4°C for 40 min before electrophoresis in the same solution at 25 V for 30 min at an ambient temperature of 4°C (temperature of running solution not exceeding 15°C). The slides were washed three times for 5 min each with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) at 4°C before staining with 20 μl 4′,6-diamidine-2-phenylindole dihydrochloride (5 μg/ml).

The comet assay as described detects strand breaks in DNA. The presence of breaks allows supercoiled loops of DNA to relax and move out to form a tail (hence, the name comet assay), and the fraction of DNA in the tail reflects the frequency of breaks (18). A modification of the assay permits detection of oxidized bases in addition to breaks (17). In this case, following lysis, the slides were washed three times for 5 min each in endonuclease III buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0), drained, and the agarose covered with 50 μl of either endonuclease III in buffer (1 μg protein/ml) or buffer only, sealed with a coverslip, and incubated for 30 min at 37°C. Endonuclease III introduces breaks in the DNA at sites of oxidized pyrimidines. Alkaline treatment, electrophoresis, and neutralization followed as before.

Quantitation of Comet Assay. Quantitation is achieved by visual scoring of 100 randomly selected comets per slide, classifying them into five categories representing different degrees of damage. This method has the advantage of speed (essential when population screening demands processing of many samples); it is calibrated by reference to computer image analysis based on fluorometric measurement of DNA intensities in head and tail (19). The arbitrary units shown in figures are the result of visual scoring, and are related (approximately linearly at the levels of damage seen here) to the percentage of DNA in the tail (19).

Plasma Antioxidants. Plasma was prepared from heparinized whole blood (10 ml) by centrifuging at 2000 × *g* for 15 min at 4°C. The samples were immediately snap frozen in liquid nitrogen and stored at −80°C. All plasma was prepared and frozen within 1 h of blood collection. Where samples were intended for vitamin C determination, an equal volume of 10% (v/v) metaphosphoric acid was added to the plasma before freezing. Vitamin C concentration was determined according to the method of Ross (20). Plasma concentrations of retinol, α-tocopherol, and γ-tocopherol and the carotenoids α-carotene, β-carotene, β-cryptoxanthin, lycopene, phytofluene, and lutein/zeaxanthin were measured according to the method of Hess *et al.* (21).

Statistics. ANOVA and Student's *t* test were carried out as appropriate, using the statistical package Genstat.

RESULTS

Endogenous DNA Damage in Relation to Antioxidant Status. Table 1 gives the mean plasma concentrations of vitamin C, α-tocopherol, and β-carotene. At the start of the trial, smokers had lower levels of vitamin C and β-carotene than nonsmokers ($P<0.003$ and $P<0.03$, respectively).

DNA strand breaks and oxidized pyrimidines were measured in lymphocytes using the comet assay. Mean values of endogenous DNA damage for subjects in the placebo groups were examined for any correlation with the individual plasma antioxidant levels, similarly averaged from the four determinations made during the trial (Table 2). A positive correlation is seen between strand breaks and α-tocopherol in nonsmokers but not smokers. Almost all of the correlations between oxidized bases and antioxidant concentrations are weakly negative; the only significant associations are seen with retinol (nonsmokers) and lycopene (smokers). The multiple correlation coefficients of strand breaks or oxidized bases with antioxidant concentrations were not significant.

Plasma Antioxidant Levels: Effect of Supplementation. Table 1 gives the mean plasma concentrations of antioxidants after supplementation for 20 weeks. Both smokers and nonsmokers demonstrated substantial increases in plasma concentrations of all three supplemented antioxidants by the end of the supplementation program.

Modulation of Endogenous DNA Damage by Supplementation. DNA strand breaks were assayed at 0, 5, 10, and 20 weeks and oxidized pyrimidines at 5, 10, and 20 weeks after the start of the trial. Fig. 1 shows the values for strand breaks, for smokers and nonsmok-

Table 2 *DNA damage correlated with plasma antioxidant concentrations*[a]

	Correlation coefficient (*r*)			
	DNA strand breaks		Oxidized pyrimidines	
Antioxidant	Smokers	Nonsmokers	Smokers	Nonsmokers
Vitamin C	+0.02	−0.15	−0.03	−0.08
α-Tocopherol	0.00	+0.46[b]	−0.04	−0.08
β-Carotene	−0.30	−0.17	−0.20	−0.05
γ-Tocopherol	+0.01	−0.03	+0.10	−0.02
Retinol	+0.01	+0.02	−0.21	−0.43[b]
α-Carotene	0.00	−0.05	−0.18	+0.01
β-Cryptoxanthin	+0.22	+0.29	+0.08	−0.34
Lycopene	+0.29	−0.03	−0.50[b]	+0.06
Phytofluene	+0.19	+0.10	−0.37	−0.19
Lutein/zeaxanthin	+0.33	+0.10	+0.10	−0.10

[a] Antioxidant concentrations were determined on the placebo groups of smokers and nonsmokers on four occasions during the trial. The means of the four determinations were assessed against mean values for strand breaks (four determinations) or oxidized pyrimidines (three determinations). The sample size varied between 18 and 25. Full details of antioxidant concentrations will appear elsewhere.

[b] Significant correlations are indicated, $P < 0.05$.

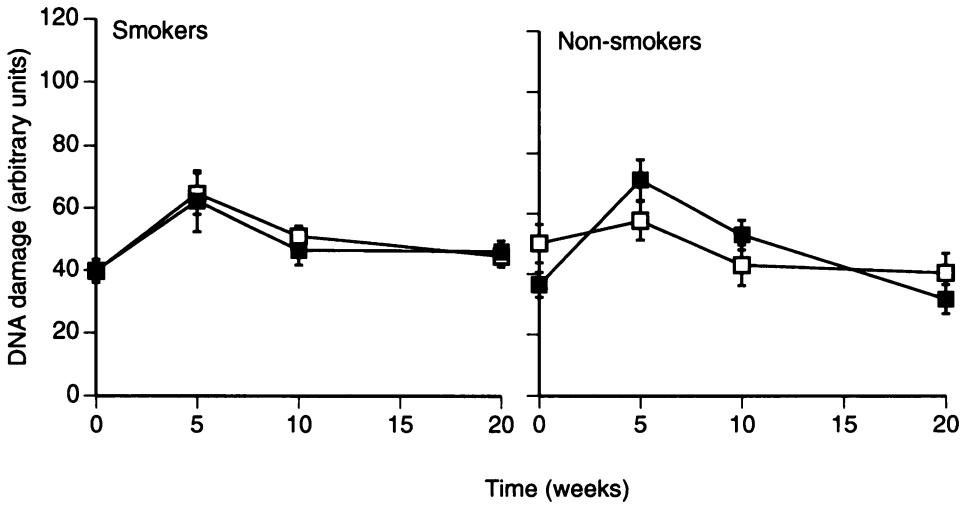

Fig. 1. DNA strand breaks measured by SCGE in lymphocytes of smokers and nonsmokers receiving placebo (□) or antioxidant supplement (■). *Bars*, SEM.

ers. There is no effect of supplementation on strand break levels; the only significant difference is a slight increase in strand breakage in smokers compared with nonsmokers at week 20 (supplement and placebo groups combined, $P<0.02$). The data for oxidized pyrimidines are presented in Fig. 2. Early in the trial, smokers had a significantly higher level of oxidized bases ($P<0.002$) than nonsmokers. After 20 weeks of supplementation, the differences between smokers and nonsmokers had disappeared, and smokers and nonsmokers taken together show a highly significant effect ($P<0.002$) of antioxidants on the level of base oxidation; the mean for the placebo group (in arbitrary units of damage) is 60.5 ± 7.0 (SE) compared with the mean for the supplemented group of 38.0 ± 5.9 (SE).

Resistance of Lymphocytes to Exogenous DNA Damage. If the antioxidant status of lymphocytes is enhanced by supplementation, they would be expected to show increased resistance to oxidative damage when challenged *in vitro* with an agent such as H_2O_2. We tested lymphocytes from randomly selected subsets of nonsmoking volunteers after 20 (or in some cases 40) weeks of administering antioxidants or placebo. They were treated with different doses of H_2O_2 and immediately processed by SCGE to determine the extent of DNA strand breakage induced (Fig. 3). Damage is significantly decreased in the supplemented samples given treatment with 0.1 mM H_2O_2 ($P<0.001$) or 0.3 mM H_2O_2 ($P<0.001$). The mean values of plasma antioxidants, measured at the time of the *in vitro* challenge, are shown for both subsets in Table 3.

DISCUSSION

The aim of this study was two-fold: first, to look for correlations between natural (*i.e.*, unsupplemented) levels of antioxidants and various indicators of genetic damage; and, second, to see whether antioxidant supplementation could significantly affect these markers. To examine the relationship between oxidative DNA damage and preexisting antioxidant status (without supplementation), the data obtained from samples within the placebo group, taken at intervals during the study, were combined to calculate mean values for the whole period in relation to both DNA damage and antioxidants. This longitudinal approach was considered preferable to the snapshot view which would have been provided by assessing all volunteers (supplement and placebo groups) once before the start of the trial, since it may help to accommodate random or seasonal variations in both the diet and the assays.

The alkaline comet assay, as used here, detects strand breaks and oxidized pyrimidines. As an index of endogenous oxidative damage to DNA, strand breaks are unreliable. The steady-state level of breaks is expected to be very low, since they can be rejoined very quickly by cellular repair (22). Furthermore, under the conditions of the assay, apurinic/apyrimidinic sites, which are alkali labile, will be revealed as breaks. Finally, both apurinic/apyrimidinic sites and actual strand breaks occur as intermediates in the cellular processing of a variety of DNA lesions via either base excision repair or nucleotide excision

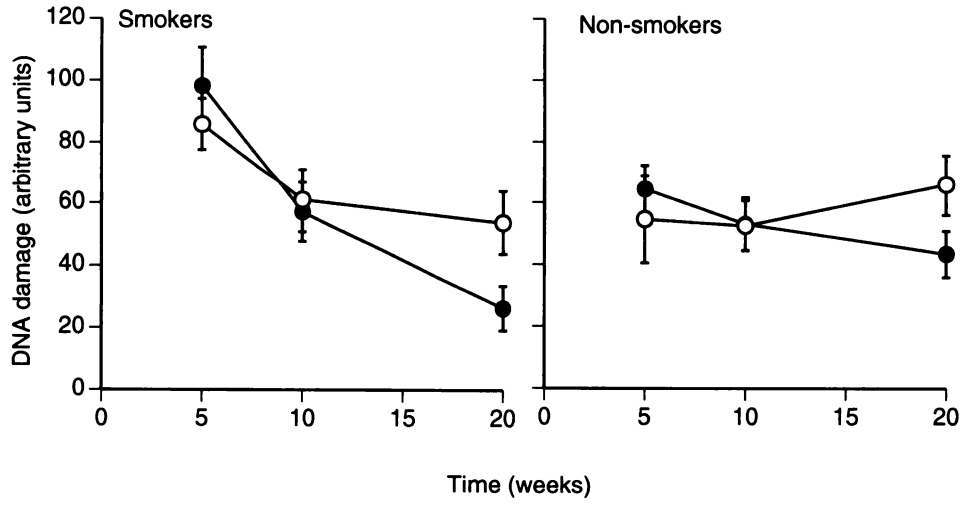

Fig. 2. Oxidation of pyrimidines in DNA of lymphocytes from smokers and nonsmokers receiving placebo (○) or antioxidant supplement (●). Pyrimidine oxidation is estimated after SCGE as the difference between DNA damage detected with endonuclease III digestion and damage detected without digestion. *Bars*, SEM.

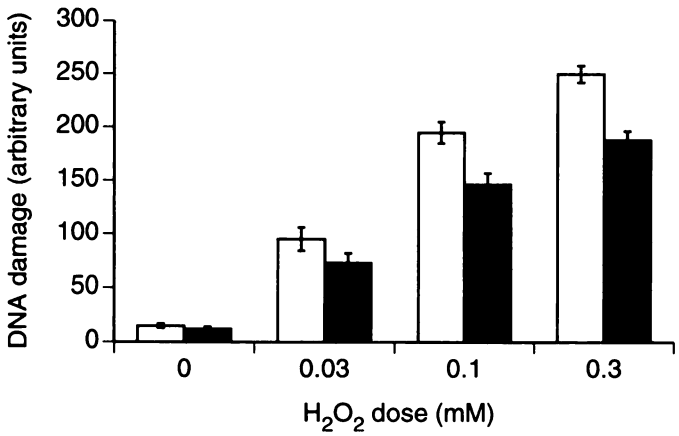

Fig. 3. *Ex vivo* test for antioxidant protection. Lymphocytes from subjects (nonsmokers) receiving placebo (□) or supplement (■) were challenged *in vitro* with H_2O_2, and DNA strand breaks were measured by SCGE. *Bars*, SEM; n = 24 (placebo), n = 17 (supplement).

repair. Thus, the positive correlation between DNA strand breaks and α-tocopherol (Table 2) should not be interpreted as indicating any deleterious effect of the vitamin. With the modified comet assay incorporating a damage-specific enzyme, endonuclease III, oxidized pyrimidines are quantitatively detected (17). These lesions, in contrast to frank breaks, are an unambiguous indicator of endogenous oxidative damage. Early in the trial, smokers showed high levels of oxidized bases compared with nonsmokers, but this difference is not seen at later times in the placebo group, and the biological significance of the observation is not clear. It may possibly reflect a seasonal effect unrelated to diet.

Table 2 shows that signs of any association between baseline (*i.e.*, unsupplemented) plasma levels of individual antioxidants and base oxidation are weak and sporadic. In a parallel study of *hprt* mutations in lymphocytes from these same volunteers (23), we found a strong negative correlation between mutant frequency and baseline plasma vitamin C concentration in smokers, but otherwise no significant correlations with antioxidant levels. The paucity of correlations is not altogether surprising, since there are many other variables that could affect endogenous DNA damage, including intrinsic antioxidant defenses, and DNA repair. Antioxidants assessed individually do not accurately reflect the overall antioxidant status; estimation of multiple correlation coefficients from the data of Table 2 is inadequate without knowledge of the weightings that need to be given to the different antioxidants depending on their biological activities. In addition, it is possible that the range of plasma antioxidant levels in our population group, reflecting the low intake of fruit and vegetables in this region (24), is too narrow to see such correlations. A comparison to a population consuming a Mediterranean diet with a high fruit and vegetable content might therefore be instructive.

The data representing the effects of supplementation are far more informative. First, as expected, there were significant and, in some cases, substantial increases in plasma antioxidant concentrations at week 20 compared with week 0 in supplemented subjects. Smokers had lower concentrations of vitamin C than nonsmokers at the start of the trial. The effect of supplementation on vitamin C levels appears to be a doubling of plasma concentration at week 20 compared with week 0, but in nonsmokers there is actually little difference between those on placebo and those on supplement at this time. The increase in vitamin C concentration in nonsmokers on placebo may represent a seasonal increase in intake of fruit and vegetables, since the study began in April and ended in September. The seasonal rise might therefore have minimized the impact of vitamin C supplementation.

The occurrence of oxidized pyrimidines in lymphocyte DNA was used as a specific index of oxidative damage. There was no difference in this parameter between subjects on supplement and those on placebo (either smokers or nonsmokers) at week 10, but by week 20 there was a very significant decrease in oxidized pyrimidines in smokers and nonsmokers receiving supplement compared with those on placebo. This indicates protection of DNA by antioxidants in the supplement against attack by endogenously produced reactive oxygen species. Fraga *et al.* (25) measured 8-hydroxydeoxyguanosine as a marker of oxidative damage in DNA of human sperm. The level of damage was significantly increased following depletion of dietary vitamin C and decreased on subsequent supplementation with vitamin C. Our study made use of a cocktail of antioxidants, and therefore it is not possible to attribute protective effects to individual compounds; it is, however, unlikely that vitamin C is responsible for the decrease in damage, since there is so small a difference in plasma concentrations between nonsmokers on supplement or placebo at this time.

The other method used here to show antioxidant protection depends on challenging lymphocytes *in vitro* with H_2O_2. In this case, strand breaks are a valid measure of incident damage, and cellular repair is not a factor because the cells are kept cold during the brief treatment. The effect of supplementation is again striking, with a significant decrease in damage received at the higher H_2O_2 doses tested. A related effect has been reported by Green *et al.* (26), who treated human lymphocytes *in vitro* with ionizing radiation. The response, in terms of DNA strand breakage, was significantly reduced in samples of blood taken a short time after a high single dose of vitamin C, compared with samples from the same subjects before ingestion of the antioxidant.

The molecular epidemiology approach is dependent on the selection of reliable and appropriate biomarkers, which ideally should have a well-established causal connection with the disease under investigation. It is generally recognized that DNA damage, resulting in base change and mutation when replication occurs, is a very early event in carcinogenesis. It seems likely that a one third fall in the level of oxidative base damage would be translated into a significantly lower risk of mutation and cancer. Such an extrapolation is justified only if peripheral nondividing lymphocytes can be regarded as a satisfactory surrogate for the actual target cells in the process of carcinogenesis, or at least as a valid indicator of the level of oxidative damage in the body overall. Application of the comet assay to other cell types such as buccal epithelial cells, urothelial cells isolated from urine, or cells isolated from biopsy material will confirm whether this is the case.

Herein, we here show for the first time a highly significant moderating effect of long-term antioxidant supplementation on endogenous and exogenous oxidative DNA damage in lymphocytes, supporting the hypothesis that dietary antioxidants may protect against cancer. The ability to demonstrate such clear differences between subjects on placebo and on supplements serves to confirm the comet assay as a useful tool in screening populations for genotoxic effects.

Table 3 *Plasma antioxidant levels in nonsmokers sampled at week 20 or week 40 for the in vitro lymphocyte challenge experiment described in Fig. 3*[a]

	α-Tocopherol/cholesterol (μM/mM)	β-Carotene (μg/ml)	Vitamin C (μM)
Placebo	5.30 ± 0.38 (n=21)	0.36 ± 0.06 (n=21)	38.3 ± 3.6 (n=23)
Supplement	7.93 ± 0.77 (n=12)	2.19 ± 0.26 (n=12)	53.2 ± 4.0 (n=16)
	P<0.002	$P<10^{-9}$	P<0.01

[a] Mean values ± SE; P values refer to differences between placebo and supplemented groups for each antioxidant.

ACKNOWLEDGMENTS

We thank Professor W. P. T. James, Dr. J. R. Arthur, and Dr. G. G. Duthie for valuable help in planning this project and in critically reviewing the manuscript; BASF for their generosity in supplying the supplements; V. L. Dobson and L. K. Crosley for skilled technical assistance; K. M. Brown for help with blood sampling; D. Hirst (Scottish Agriculture Statistics Service) for the statistical analysis of the data; and Dr. J. Beattie and colleagues of Inverurie Medical Group and all of the volunteers for their enthusiasm and commitment to the trial.

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