Effect of Dietary Vitamin E on Spontaneous or Nitric Oxide Donor-Induced Mutations in a Mouse Tumor Model

Jagdeep K. Sandhu, Arsalan S. Haqqani, H. Chaim Birnboim

Background: Vitamin E, an antioxidant, has been investigated for its effect on cancer incidence in humans, but no firm conclusions about a protective effect can be drawn from these studies. Recently, we reported a statistically significant correlation in the Mutatect mouse tumor model between the number of neutrophils and the frequency of mutation at the hypoxanthine phosphoribosyltransferase (hppt) locus. We have now used this model to investigate vitamin E’s effect on the hppt mutation rate. Methods: Mutatect cells were grown in mice as subcutaneous tumors for 2–3 weeks, the tumor cells were recovered, and 6-thioguanine-resistant (i.e., hppt mutant) colonies were scored. Myeloperoxidase activity was used as a measure of neutrophil infiltration. Vitamin E (2 IU/kg body weight) was provided in the diet for 3–4 weeks. In some experiments, glyceryl trinitrate (100 mg/kg body weight) was also administered as a source of nitric oxide. All statistical tests were two-sided. Results: Mouse tumors from the Mutatect MN-11 cell line exhibited a 3.2-fold higher median mutation frequency than the same cells in culture (P < .0001); vitamin E reduced this frequency by 24.9% (P = .01). Mutatect TM-28-derived tumors (which secrete interleukin 8) were heavily infiltrated with neutrophils and had a correspondingly high mutation frequency; in two separate experiments, vitamin E reduced the median mutation frequency by 68.9% (P = .0019) and 84.1% (P = .011) and myeloperoxidase levels by 75.3% (P = .0002) and 75.5% (P = .026), respectively. Glyceryl trinitrate increased the mutation frequency in MN-11 tumors, and vitamin E reduced the median frequency by 61.4% (P = .058). Conclusions: Dietary vitamin E afforded strong protection against both spontaneously arising and nitric oxide-induced mutations. Two separate protective mechanisms by vitamin E may be operating: scavenging of a nitric oxide-related genotoxic species and altering the infiltration of neutrophils into tumors. [J Natl Cancer Inst 2000;92: 1429–33]

Vitamin E is the most important lipophilic antioxidant in the prevention of cellular injury associated with oxidative stress (1–4). In natural dietary sources, vitamin E occurs as a mixture of many different tocopherols and tocotrienols, of which d-α-tocopherol is to be considered the major biologically active form. Dietary supplements of vitamin E, usually a different mixture than that occurring in foods, are widely self-administered in the belief that they can protect against cardiovascular disease and cancer. However, clinical trials of vitamin E supplements or studies of disease risk associated with serum vitamin E levels have failed to show any consistently strong anticancer effect, except perhaps against prostate cancer (5–12).

Many types of chronic inflammatory conditions in humans predispose to malignancy (13–15). Neutrophils, monocytes, and macrophages, present at sites of inflammation, generate a variety of oxidants and nitrosants, which, as a class, are known to be genotoxic and mutagenic (16,17). Similar cell types are often found in solid tumors. Using a mouse tumor model, we have recently shown (18) that the number of infiltrating neutrophils and the level of nitric oxide synthase correlate positively with an increase in hypoxanthine phosphoribosyltransferase (hppt) mutation frequency in the tumor cells, suggesting that neutrophil-derived reactive oxygen and nitrogen species were involved. The Mutatect model is a series of related cell lines that can be grown as subcutaneous tumors in syngeneic C57BL/6 mice; the system permits ready detection of mutations arising in vivo (19). This model was the first to show a quantitative increase in mutation frequency as a result of chronic administration of vitamin E...
of factors in the tumor microenvironment when compared with the same cells in culture (20). We now report on the antimutagenic effect of dietary vitamin E in this experimental model.

**Materials and Methods**

Cells and culture conditions. Mutatect cells were cultured in nonselective medium (Dulbecco’s modified Eagle medium with 10% fetal calf serum), HAT medium (supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 15 μM thymidine), HT medium (without aminopterin), or 6-TG medium (supplemented with 50 μM 6-thioguanine), as described previously (20). Before use, Mutatect cells were grown in HAT medium for 7 days to kill any pre-existing mutants, and then they were transferred to HT medium for 2 days to allow recovery from HAT treatment.

Construction of an interleukin 8 (IL-8)-expressing Mutatect TM-28 cell line. Human IL-8 complementary DNA (cDNA) #38322 from L.M.A.G.E. Consortium (Lawrence Livermore National Laboratories, Livermore, CA) was used as the starting material (21). Errors in the cDNA were corrected, and the cDNA was cloned into vector pTRE (Clontech Laboratories, Inc., Palo Alto, CA) to create pTRE-IL8. Tetracycline-responsive transcriptional activator pTET-off (Clontech Laboratories, Inc.) was stably transfected into Mutatect MC-TGS17-51 cells to create the MT-6 cell line. pTRE-IL8 was introduced into the MT-6 cells, and clone TM-28 was selected. The TM-28 clone produces biologically active IL-8, as determined by an in vitro chemotaxis assay for human neutrophils (Huaqani AS, Sandhu JK, Birnboim HC: unpublished data).

Mutatect tumor formation, detection of mutants, and myeloperoxidase measurement. Tumors from Mutatect MN-11, MT-6, and TM-28 cells were established by subcutaneous injection of cells into the flanks of C57BL/6 female mice, 8–10 weeks of age (Charles River Laboratories, Quebec, Canada). Tumors were excised when they reached 1 cm in size (2–3 weeks, depending on the cell line); single-cell suspensions were prepared by gentle mechanical dispersion of the tumor fragments with a plastic syringe. Tumor cells were established in culture for 2–4 days, and the frequency of mutations arising in vivo was then estimated from the number of colonies capable of growth in 6-TG medium, as described earlier (20). Mutation frequency is expressed as the number of 6-thioguanine-resistant colonies per 10^5 clonable tumor cells. The same tumor cell suspension was also used for measurement of myeloperoxidase activity, a marker of neutrophil infiltration (22).

Vitamin E supplements to the diet. Dietary vitamin E (d-alpha-tocopherol acetate; Novartis, Mississauga, ON, Canada) was administered by diluting it in soy oil and adding the liquid to dry standard rodent chow (Charles River Laboratories). Animals were isolated, one per cage, to ensure that the pellet was consumed. Control animals were treated similarly. Vitamin E supplements were started 7 days before tumor cell injection and were continued until the animals were killed (i.e., a total of 3–4 weeks, depending on the cell line). Control animals received 0.45 IU of vitamin E per day from their standard chow (90 IU of vitamin E/kg chow). Vitamin E-supplemented animals received an additional 2 IU of vitamin E (α-tocopherol acetate) per mouse per day (400 IU vitamin E/kg chow); they were estimated to have also received 0.50 mg of γ-tocopherol and 0.25 mg δ-tocopherol from the 20 μL of soybean oil used as a vehicle. Control animals received neither vehicle nor supplementary tocopherol. Experiments were carried out at the Animal Care and Veterinary Service of the University of Ottawa in accordance with guidelines of the Canadian Council on Animal Care. Vitamin E determinations were carried out with the use of a high-performance liquid chromatography method (23).

Induction of mutations by glyceryl trinitrate and by molsidomine in MN-11 tumor-bearing animals. On day 12 after injection of MN-11 cells, mice received an intraperitoneal injection of either injectable glyceryl trinitrate (Saxbe, Boucherville, Quebec) or molsidomine (Hoechst Marion Roussel Canada, Laval, Quebec). Control mice were given an injection of phosphate-buffered saline. Two days after treatment, tumors were recovered and established in culture as described above. Cells were cultured for 8 days to allow expression of the mutant phenotype before challenge with 6-thioguanine.

**Statistical analyses.** Nonparametric tests were used for the statistical analyses. Two groups were compared by use of the Mann–Whitney U test. Correlation between two variables was determined with the use of the Spearman rank coefficient. All P values shown are two-tailed. A P value of <.05 was considered to be statistically significant, and a P value of <.01 was considered to be highly statistically significant. Confidence intervals (CIs) of median differences were calculated with the use of Analyse-it Version 1.5 (www.analyse-it.com). Other statistical calculations were done with the use of GraphPad Prism Version 3 (GraphPad Software, San Diego, CA; www.graphpad.com).

**Results**

Effect of vitamin E on spontaneous mutation frequency in MN-11 tumors. The mutation frequency was estimated as the number of hprt mutant colonies. We have shown earlier (20) a fourfold increase in mutation frequency in cells recovered from tumors as compared with the same cells grown in vitro for the same period. This observation was confirmed and extended with the use of a larger series of tumors (Table 1). Results from a total of 197 tumors analyzed over a 4-year period are presented. The distribution of mutation frequency in tumors was non-Gaussian; therefore, nonparametric tests were used for statistical analysis. In agreement with earlier results, these data show a 3.2-fold increase in median mutation frequency in MN-11 cells recovered from tumors as compared with the same cells maintained in culture (P<.0001). We have recently shown (18) that there exists a positive correlation (r = .63; P<.0001) between the number of infiltrating neutrophils found in MN-11 tumors and the mutation frequency of cells recovered from these tumors. Neutrophils contained inducible nitric oxide synthase, and the mutation frequency was shown to be correlated with nitric oxide synthase activity. To determine whether an antioxidant, vitamin E, might affect the mutation frequency, we tested it as a dietary supplement.

The results shown in Table 2 indicate that supplementation of standard chow (which contains 90 IU vitamin E/kg chow) with 400 IU vitamin E/kg chow for 3 weeks reduced the median mutation frequency by 24.9% (P = .01).

High level of myeloperoxidase and high mutation frequency in IL-8-producing TM-28 tumors. IL-8 is a potent chemoattractant for neutrophils. We have shown elsewhere that direct intratumoral injection of human IL-8 increased both the neutrophil content and the mutation frequency of MN-11 tumors (18). To provide further evidence that increased neutrophil content can affect mutation frequency, we constructed a cell line that secretes human IL-8, Mutatect TM-28. Fig. 1, A, shows that TM-28 tumors contained high levels of myeloperoxidase, a neutrophil-specific marker (22), as compared with Mutatect MT-6 tumors, the non-IL-8-expressing parental

| Table 1. Elevated frequency of spontaneously arising hprt mutated cells in Mutatect MN-11 cells grown as tumors compared with cells in culture* |
|-----------------|-------|-----------------|-----------------|
| Median mutation frequency (25–75 percentiles) | No. | Difference between medians (95% confidence interval) | Mann–Whitney U test for difference between medians |
| Cells in culture | 6.0 (4.5–9.0) | 55 | 12.8 (9.4–16.4) | P = .0001 |
| Tumors | 19.3 (11.2–33.1) | 197 | |

*Tumors were allowed to form for 14–16 days before mutation frequency was determined. Mutation frequency is expressed as the number of 6-thioguanine-resistant colonies per 1 × 10^5 clonable tumor cells.
Table 2. Effect of vitamin E on the frequency of spontaneously arising hprt mutant cells in Mutatect MN-11 tumors*

<table>
<thead>
<tr>
<th>Diet</th>
<th>Median mutation frequency (25–75 percentiles)</th>
<th>No.</th>
<th>Difference between medians (95% confidence interval)</th>
<th>Mann-Whitney U test for difference between medians</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard diet</td>
<td>27.0 (17.9–62.2)</td>
<td>21</td>
<td>11.9 (3.0–26.6)</td>
<td>$P = .01$</td>
</tr>
<tr>
<td>Vitamin E diet</td>
<td>20.3 (10.6–24.6)</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mutation frequency is expressed as the number of 6-thioguanine-resistant colonies per $1 \times 10^5$ clonable tumor cells.

Fig. 1. Effect of dietary vitamin E on the mutation frequency and myeloperoxidase levels in Mutatect TM-28 tumors. TM-28 is a Mutatect cell line that expresses human interleukin 8, and MT-6 is its parental line. Mutation frequency is expressed as the number of 6-thioguanine-resistant colonies per $1 \times 10^5$ clonable tumor cells. Units of myeloperoxidase activity are as in (22). A) TM-28 tumors show a higher mutation frequency and myeloperoxidase activity than MT-6 tumors. B) TM-28 tumors from animals receiving vitamin E (+vit E) supplements have a lower mutation frequency than those from animals on control diets (−vit E). Each panel represents a separate experiment. C) TM-28 tumors from animals receiving vitamin E supplements have lower corresponding myeloperoxidase activity than those from animals on control diets. Different scales are used on the left and right axes in panels B and C to permit an easier comparison of the data from the two experiments. D) Replot of data from panels B and C, showing correlations between mutation frequency and myeloperoxidase activity in individual tumors. Squares and triangles represent the results of two separate experiments, approximated by the two regression lines. In panels B–D, the open symbols represent vitamin E-fed animals and the filled symbols represent control animals. Each symbol represents a tumor from a single animal. Where the symbols overlap in panel A, the number of tumors is six (both left and right); the horizontal line in panels A–C represents the median. Other details are as described in the “Materials and Methods” section, except that the in vivo growth of subcutaneous tumors was for 3 weeks.

Decrease in myeloperoxidase and mutation frequency by vitamin E in TM-28 tumors. Administration of vitamin E for 4 weeks was tested in Mutatect TM-28 tumors for its effect on mutation frequency and myeloperoxidase. The plasma level of vitamin E was 2.36 ± 2.26 μg/mL (mean ± standard deviation [SD], n = 8) in the control group fed standard chow and 3.21 ± 0.74 μg/mL (mean ± SD, n = 8) in the vitamin E-supplemented group ($P = .021$). The data shown in Fig. 1, B, indicate that vitamin E supplementation had a marked effect on mutation frequency. In two separate experiments, the median mutation frequency of the vitamin E group was statistically significantly lower than that of the control group: 68.9% lower in experiment 1 ($P = .0019$) and 84.1% lower in experiment 2 ($P = .011$). (For experiment 1, the difference between medians = 61.0 [95% CI = 23.0–108.0]; for experiment 2, the difference between medians = 222.0 [95% CI = 64.0–772.0].) Tumor-infiltrating neutrophils, as assessed by myeloperoxidase activity, were similarly lower (Fig. 1, C). Myeloperoxidase levels were 75.3% lower in tumors from vitamin E-supplemented animals compared with the control group in experiment 1 ($P = .0002$) and 75.5% lower in experiment 2 ($P = .026$). For experiment 1, the difference between medians = 166.5 [95% CI = 100.0–251.0]; for experiment 2, the difference between medians = 39.0 [95% CI = 13.0–63.0].) The relationship between myeloperoxidase and mutation frequency in tumors from both experiments is shown in Fig. 1, D. The plots show that tumors from vitamin E-supplemented animals (open symbols) had both a lower myeloperoxidase and a lower mutation frequency than control animals (filled symbols). The mutation frequency of the control group in the two experiments (filled triangles and squares) differed by about threefold, but the reduction in mutation frequency by vitamin E was very similar (Fig. 1, B). It is interesting that the two experiments showed a markedly different relationship between myeloperoxidase and mutation frequency (Fig. 1, D); a very high myeloperoxidase level in experiment 1 (solid line) was associated with a relatively low mutation frequency, and the converse was observed in experiment 2 (Spearman rank correlation: for experiment 1, $r = .55$ and $P = .028$; for experiment 2, $r = .86$ and $P < .0001$). There was no observable difference in either myeloperoxidase or mutation frequency between control animals that received no vehicle and animals that received soy oil (data not shown).

Induction of mutations by nitric oxide-donating drugs and effect of vitamin E. Nitric oxide and related reactive nitrogen oxide species are potentially mu-

Journal of the National Cancer Institute, Vol. 92, No. 17, September 6, 2000

REPORTS 1431
trogenic (16,17). We have shown (24) that nitrovasodilator drugs, glyceryl trinitrate and sodium nitroprusside, were very effective at inducing mutations in cultured MN-11 cells. To assess the in vivo mutagenicity of such drugs, we gave MN-11 tumor-bearing mice an intraperitoneal injection of either glyceryl trinitrate or molsidomine (a chemically unrelated nitrovasodilator drug) 2 days before tumor excision. Both glyceryl trinitrate and molsidomine caused a dose-dependent increase in mutation frequency. The maximum effect was observed at the highest dose tested: Glyceryl trinitrate at a dose of 5 mg/kg induced about 130 mutations, and molsidomine at a dose of 100 mg/kg induced about 50 mutations per 1 × 10⁵ cells over spontaneous levels. Vitamin E was tested for its ability to protect against mutagenicity induced by glyceryl trinitrate (Fig. 2). Tumor cells from animals whose diets were supplemented with vitamin E had a 61.4% reduction in median mutation frequency (P = .058) (72% if the average control frequency is subtracted). (The difference between medians = 192.7 [95% CI = −10.3 to 442.5].) These results indicate that an antioxidant, vitamin E, can reduce the mutation frequency induced by glyceryl trinitrate, a drug that releases nitric oxide. No change in tumor neutrophil content following glyceryl trinitrate treatment compared with controls was seen, as determined histologically (18) (data not shown).

**DISCUSSION**

The Mutatect mouse tumor model was developed to permit the detection of mutations arising in vivo as a result of mutagenic factors in the tumor microenvironment. It has allowed us to demonstrate a positive statistical correlation between the number of tumor-infiltrating neutrophils and the frequency of mutations at the hprt locus (18). The hprt gene is non-essential, and mutational events can be readily scored on the basis of resistance to 6-thioguanine. An increase in frequency of mutations at this locus is commonly used as evidence of genotoxic insult and as a surrogate for genetic instability associated with tumor progression.

Vitamin E is an important group of lipophilic antioxidants found at high concentration in polyunsaturated vegetable oils (1). Dietary supplements usually consist of natural or synthetic α-tocopherol ester. In clinical trials, a small benefit of low-dose vitamin E supplements (50 IU per day) in fatal cardiovascular diseases has been reported (25), but evidence for its utility in reducing cancer risk has been inconclusive (9,26,27). Our report is the first, to our knowledge, to show an unequivocal protective effect of dietary vitamin E supplements (2 IU per mouse per day for 3–4 weeks) on mutation frequency in an experimental tumor—a relatively short period of dietary supplementation with vitamin E (d-α-tocopherol acetate) was able to reduce markedly the level of mutant cells arising in vivo. In a transgenic mouse model designed to study spontaneously arising mutants in different normal tissues, 3 months of dietary supplementation with vitamin E (4.1 IU per mouse per day) failed to detectably reduce the mutation frequency at a lacI transgenic marker locus (28). This striking difference strongly implies that the mechanism of induction of spontaneously arising mutations in normal tissues is different from spontaneously arising mutations in a tumor, presumably attributable to factors in the tumor microenvironment.

Our data are best explained by posulating a dual action of vitamin E. Vitamin E acts as a scavenger of nitric oxide or other reactive nitrogen oxide species, since it strongly inhibited glyceryl trinitrate-induced mutagenesis in the absence of any change in neutrophil content (Fig. 2). A second mechanism is suggested by the results shown in Fig. 1, where tumor cell suspensions from vitamin E-supplemented mice had a markedly lower level of myeloperoxidase (a neutrophil-specific marker) than those from control mice. The latter observations suggest that vitamin E may affect the number or distribution of neutrophils in tumors, possibly by altering the expression of cell adhesion molecules. A clinical study supports the latter possibility. Vitamin E (600 IU) given for 8 days to patients prior to surgery for aortic abdominal aneurysm attenuated the influx of neutrophils associated with ischemia-reperfusion injury of the lower limbs (29). The level of E-selectin (a cell adhesion molecule) and ICAM-1 (i.e., intercellular adhesion molecule-1), molecules responsible for adhesion of circulating neutrophils to endothelium, was reported to be lower in patients receiving vitamin E. Changes in neutrophil distribution and adhesion after vitamin E supplementation are currently being explored in the Mutatect model. Although we have also shown an in vitro protective effect of vitamin E on glyceryl trinitrate-induced mutagenesis (unpublished data), the possibility of a second mode of action of vitamin E could not have been predicted without the use of this animal model.

A strong positive correlation between the number of tumor-infiltrating neutrophils (measured by direct counting in histologic sections or by the level of myeloperoxidase in tumor homogenates) and the hprt mutation frequency was consistently seen in this report (Fig. 1, D) and in another published study from our laboratory (18). However, in both cases, the slope of the correlation line between mutation frequency and the absolute neutrophil count and/or level of myeloperoxidase in tumor homogenates) and the hprt mutation frequency was consistently seen in this report (Fig. 1, D) and in another published study from our laboratory (18). However, in both cases, the slope of the correlation line between mutation frequency and the absolute neutrophil count and/or level of myeloperoxidase in tumor homogenates) and the hprt mutation frequency was consistently seen in this report (Fig. 1, D) and in another published study from our laboratory (18).

[Image of Fig. 2: Effect of vitamin E (VitE) on the frequency of mutations induced by glyceryl trinitrate (GTN). Mutation frequency is expressed as the number of 6-thioguanine-resistant colonies per 1 × 10⁵ clonable tumor cells. Mutatect MN-11 cells (5 × 10⁴) were injected into three groups of mice at day 0. The control group was given an injection of phosphate-buffered saline, and the GTN groups were given an intraperitoneal injection of GTN (5 mg/kg) on day 12. The GTN + VitE group in addition received a dietary supplement with α-tocopherol acetate (100 mg/kg per day, starting at day −7). The respective median mutation frequencies were 34.1, 255.0, and 98.5 in control, GTN, and GTN + VitE groups. The difference between the GTN and GTN + VitE groups is not quite statistically significant (P = .058). No difference was seen histologically in neutrophil content as a result of GTN treatment. Each symbol represents a single tumor; the horizontal line represents the median.]

**Fig. 2.** Effect of vitamin E (VitE) on the frequency of mutations induced by glyceryl trinitrate (GTN). Mutation frequency is expressed as the number of 6-thioguanine-resistant colonies per 1 × 10⁵ clonable tumor cells. Mutatect MN-11 cells (5 × 10⁴) were injected into three groups of mice at day 0. The control group was given an injection of phosphate-buffered saline, and the GTN groups were given an intraperitoneal injection of GTN (5 mg/kg) on day 12. The GTN + VitE group in addition received a dietary supplement with α-tocopherol acetate (100 mg/kg per day, starting at day −7). The respective median mutation frequencies were 34.1, 255.0, and 98.5 in control, GTN, and GTN + VitE groups. The difference between the GTN and GTN + VitE groups is not quite statistically significant (P = .058). No difference was seen histologically in neutrophil content as a result of GTN treatment. Each symbol represents a single tumor; the horizontal line represents the median.
recruitment and/or the state of activation of the neutrophils.

Chronic inflammatory conditions, such as ulcerative colitis, Crohn’s disease, Schistosomiasis, and Helicobacter pylori infection (13–15), are important risk factors for cancer, possibly because of mutagenic reactive oxygen and nitrogen species generated by inflammatory cells. Rheumatoid arthritis is a chronic inflammatory disease associated with malignancy (31), in which a high frequency of mutations has been found (32). Vitamin E may protect against the development of cancers associated with inflammatory conditions by scavenging these reactive species. However, an additional mechanism by which vitamin E may be protective is suggested by the results presented in Fig. 1: Once tumors become established, vitamin E may reduce mutations by affecting the infiltration of neutrophils or other inflammatory cells.

In summary, our results suggest that vitamin E may exert antmutagenic/anticancer properties by two distinct mechanisms. Both of these mechanisms should be taken into account in the planning of future clinical trials.

REFERENCES


NOTES

Supported by grants from the Cancer Research Society and the Medical Research Council of Canada to H. C. Birnboim. A. S. Haqqani is supported by a Doctoral research award from the Medical Research Council of Canada. H. C. Birnboim is a Senior Cancer Scientist of Cancer Care Ontario. We thank Donna Grant and Denise Proulx for their skillful technical assistance. We also thank Dr. Nick Hidiroglou and Rene Madere of the Nutrition Section, Health Canada, for performing plasma vitamin E analyses.

Manuscript received January 28, 2000; revised June 7, 2000; accepted June 20, 2000.