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Carcinogen-Induced Chromosomal Breakage Decreased by Antioxidants

(sodium cyclamate/7,12-dimethylbenz(α)anthracene/peroxidation/aging)

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ABSTRACT **Blood leukocyte cultures were incubated with antioxidants and the carcinogens sodium cyclamate and 7,12-dimethylbenz(α)anthracene in different combinations. There were 17.4% more chromosomal breaks in the group of cells treated with dimethylbenzanthracene only than in the untreated controls. The reductions in chromosomal breaks by the antioxidants were as follows: ascorbic acid, 31.7%; butylated hydroxytoluene, 63.8%; Na_2SeO_3, 42.0%; and *dl*-α-tocopherol, 63.2%. Multiple chromosomal breaks were distributed equally throughout the experimental groups. Sodium cyclamate had only slightly more chromosomal breaks than the controls (11.6 compared to 10.9%). In the cyclamate groups treated with Na_2SeO_3, 11.2% of chromosomes were broken. More acrocentric-type chromosomal breaks (21.7%) were seen in the untreated cells than the cells treated with cyclamate (3.4%) or dimethylbenzanthracene alone (4.8%). The carcinogen-treated groups had a higher percentage of meta breaks than the untreated controls.**

The antioxidants, selenium (1–3), *dl*-α-tocopherol (1, 2), and ascorbic acid (4), applied to mouse skin, significantly reduced tumor formation induced by 7,12-dimethylbenz(α)anthracene and croton oil. Dietary selenium has decreased the number of mouse-skin tumors induced by dimethylbenzanthracene–croton oil (1), rat-liver tumors induced by *N*-2-fluorenylacetamide (5), and liver tumors induced by diethylaminoazobenzene (6). Rats fed tocopherol-rich wheat-germ oil had fewer mixed tumors (7) after intraperitoneal injection of 3-methylcholanthrene than rats fed a control diet. Haber and Wissler (8) have reported that a tocopherol-supplemented diet had an inhibitory effect on subcutaneous sarcoma from injection of methylcholanthrene in mice. The incidence of gastric cancer in mice decreased after the mice were given the potent food antioxidants, butylated hydroxyanisole and butylated hydroxytoluene (9). After application of dimethylbenzanthracene to mouse skin on day 1, an increase of peroxidation (4) was observed until day 20. When antioxidants were applied to mice treated with dimethylbenzanthracene on days 2–21, the number of tumors developed with croton oil in the mice treated with antioxidant was considerably decreased (2).

The current belief about the mechanism of carcinogenesis is that a carcinogen, virus, or other factors alter a macromolecule such as DNA, and change the inherent information that can be transmitted to daughter cells or change the encoded information needed for metabolic function and control during the life of the cell. Marked damage of DNA has been induced with H_2O_2 or the organic peroxide, ascaridole (10); H_2O_2 destroys the pyrimidine moieties of nucleotides, and has other effects on the DNA molecule (11–13). The chemical linkage of the polycyclic hydrocarbons, benzo(α)pyrene, 3-methylcholanthrene, and dimethylbenzanthracene, to calf-thymus DNA has been induced by incubation with dilute solutions of H_2O_2 (14). Because antioxidants prevent carcinogenesis in animals (1–9) and possibly prevent certain types of human cancer (15–17), antioxidants might also prevent chromosomal damage or breakage. The objective of these experiments is to see if antioxidants prevent chromosomal breakage.

MATERIALS AND METHODS

Chemicals. Bactophytohemagglutinin M was supplied by Difco Laboratories, Detroit, Mich. This solution was reconstituted before use with 5 ml of Hanks' balanced salt solution (free of calcium and magnesium), Grand Island Biological Co., Grand Island, N.Y. Chromosome medium 1A (contains antibiotics, without phytohemagglutinin) was also from Grand Island Biological Co. Dimethylbenzanthracene was purchased from Eastman Kodak, Rochester, N.Y.; it was recrystallized twice from benzene. Sodium cyclamate, *dl*-α-tocopherol, and hydroxytoluene were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium selenite came from BDH Chemicals Ltd., Poole, England. Sodium ascorbate was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio. Giemsa stain is from Fisher Scientific Co., Fair Lawn, N.J. Velban (vinblastine sulfate) was supplied by Eli Lilly and Co., Indianapolis, Ind.

Procedure. Several tubes containing about 4.5 ml of heparinized blood were drawn from a 38-year-old male volunteer each morning before breakfast throughout the experiment. A modified macrotechnique of chromosome production was then used (18). Phytohemagglutinin M, 0.2 ml, was injected through the stopper. The tubes were inverted several times, and then placed into an ice-water bath for 1 hr. The blood samples, with the rubber stoppers intact to minimize bacterial contamination, were centrifuged in a Phillips Drucker model L-708 table centrifuge at 400 rpm for 5 min. The stoppers were removed, and 2.0 ml of plasma was transferred aseptically to a tube of chromosomal medium 1A.

The tubes containing the chromosomal medium were incubated at 37 for 3 days. About 15 hr before harvest, the antioxidants and carcinogens were added separately in 0.2 ml of 2% bovine-serum albumin. Stoltz (19) observed no significant increase of chromosomal aberrations between 5 and 25 hr of carcinogenic exposure. Just before use all antioxidants

TABLE 1. *Effect of antioxidants on chromosomal breakage induced by 1.6 μM 7,12-dimethylbenz(α)anthracene*

Antioxidant + DMBA	Cells	Breaks (%)		Breaks (%) minus control	Reduction (%)
None (DMBA only)	290	82	(28.3)	(17.4)	—
Ascorbic acid (10 μM)	127	30	(23.6)	(11.9)	(31.7)
BHT (0.21 μM)	157	29	(18.4)	(6.3)	(63.8)
Na_2SeO_3 (0.20 μM)	171	37	(21.6)	(10.1)	(42.0)
dl-α-tocopherol (10 μM)	156	28	(17.9)	(6.4)	(63.2)
None	211	23	(10.9)	—	—

DMBA, 7,12-dimethylbenz(α)anthracene; BHT, butylated hydroxytoluene.

and carcinogens were dissolved in 2 drops of acetone and then suspended in 2% bovine-serum albumin.

2 Hr before harvest, 0.2 ml of Velban (0.5 μg/ml) was added to each tube and mixed thoroughly. The techniques were those routinely used for chromosomal analyses at the Cleveland Clinic. The tubes were centrifuged at 900 rpm for 5 min. The supernatant was removed, and the cells were suspended in an equal volume of freshly prepared 0.75% sodium citrate. The solution was allowed to stand for 20 min and was centrifuged again at 900 rpm. The supernatant was discarded, and the cells were suspended in an equal volume of freshly made Carnoy's solution (1 part glacial acetic acid–3 parts methanol) for 20 min. The cells were centrifuged at 900 rpm for 20 min. The cells were suspended in one or two drops of fixative. The suspension was then placed on cover slips and flame dried. The cover slips were stained with Giemsa stain (1 part filtered Giemsa–5 parts tap water) for 5–10 min. The excess stain was removed with tap water. The slides were dried and then mounted with permount on glass slides.

Chromosome Evaluation. Most of the metaphase figures evaluated contained 46 chromosomes, but in a few cases either 45 or 47 chromosomes were observed. Cells that were grossly defective or contained only a partial number of chromosomes were not evaluated. All chromosomal abnormalities were scored. Most of the aberrations were gaps and breaks. Exchange figures and unusual chromosomes were observed infrequently (less than 1%). In all experimental groups chromosomes from at least 100 metaphase figures were observed. Controls containing dimethylbenzanthracene and controls without any addition were evaluated throughout the experiment.

Antioxidant and Carcinogen Concentrations. All antioxidants were tested initially at 10 μM. For Na_2SeO_3 and hydroxytoluene, this concentration completely inhibited growth. At 1 μM, hydroxytoluene completely inhibited growth and Na_2SeO_3 was almost completely inhibitory. At about 0.2 μM, both substances gave growth equal to the controls. Dimethylbenzanthracene at 100 and 10 μM caused about a 65 and 33% reduction in the number of mitotic figures. At 1.6 μM, growth in the dimethylbenzanthracene group was equal to the controls without additives. A greater number of dimethylbenzanthracene controls resulted because only two groups containing antioxidants could be done in one experiment along with the dimethylbenzanthracene control.

RESULTS

The group of cells treated only with dimethylbenzanthracene had 17.4% more chromosomal breaks than the untreated controls. Chromosomal breakage was reduced by all of the antioxidants tested (Table 1). The reductions were as follows: ascorbic acid, 31.7%; hydroxytoluene, 63.8%; Na_2SeO_3, 42.0%; and *dl*-α-tocopherol, 63.2%. The number of cells with multiple chromosomal breaks and with either one extra or one deleted chromosome are distributed equally throughout the experimental groups. The groups of cells treated with 100 μM sodium cyclamate had only a slightly higher percentage of broken chromosomes than the controls. The cyclamate group had 26 breaks in 222 metaphase figures (11.6%). The cyclamate group containing 0.20 μM Na_2SeO_3 had 9 breaks in 80 metaphases (11.2%). The untreated group had 23 breaks in 211 metaphases (10.9%).

The types of chromosomal breaks for the untreated cells and the cells treated with dimethylbenzanthracene or cyclamate alone seemed to be somewhat different. In a preliminary experiment, about 20–29 chromosomal abnormalities were classified as meta, submeta, or acrocentric for each of the above three experimental groups. In the control group, 9 breaks were meta, 7 were submeta, 5 were acrocentric, and 2 were not classified. In the dimethylbenzanthracene group, 16 breaks were meta, 3 were submeta, 1 was acrocentric, and 1 was a translocation. In the cyclamate group, 20 breaks were meta, 7 were submeta, 1 was acrocentric, and 1 was a translocation.

DISCUSSION

The protection against chromosomal breakage provided by antioxidants may have important relationships to aging and carcinogenesis. Accumulative spontaneous alterations in chromosomal material are believed to contribute to aging (20–22). In general, the number of chromosomal aberrations in regenerating liver cells of mice increases with age (20). Free radical inhibitors such as 2-mercaptoethylamine hydrochloride, hydroxytoluene, and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline increased the mean life-span of mice, but had little, if any, effect on the maximum life-span (23–25).

The protection against chromosomal breakage by antioxidants also seems to be consistent with epidemiological evidence that antioxidants prevent cancer. An inverse relationship has also been observed between selenium exposure and mortality from cancer in the United States (15) and Canada (17). Mortality from carcinoma of the gastrointestinal tract is much lower in the high-selenium cities and shows the greatest difference in mortality from carcinomas of various types. A relationship has been postulated (16) between the declining American death rate from gastric carcinoma and the public acceptance in 1930 of wheat cereals rich in selenium and vitamin E and the introduction of food preservative antioxidants in 1947. Similarly, the high incidence of gastric carcinoma in Iceland may be related to a large intake of smoked food, perhaps in association with a low intake of vitamin C (26). Schlegel *et al.* have observed that vitamin C reduces uroepithelial carcinoma in mice and have postulated a similar mechanism in humans (27).

Our 15-hr incubation is considerably shorter than the

24-hr incubation used in most experiments where carcinogens break chromosomes. Artifactual production of peroxide during the incubation might break chromosomes. This possibility is unlikely because carcinogens also damage chromosomes *in vivo*. In addition our incubations are done in stoppered tubes without agitation in the dark. Antioxidants may also prevent the activation of various carcinogens to epoxides (28). K-Region epoxides derived from benz(α)anthracene and dimethylbenzanthracene are more active in the malignant transformation of hamster-embryo cells than the hydrocarbons or the corresponding K-region phenols.

Although our finding that antioxidants prevent chromosomal breakage in tissue culture is consistent with carcinogenic experiments in animals, biochemical studies, and epidemiological studies, the anticarcinogenic effect in tissue culture cannot be differentiated from mutagenic, teratogenic, or cellular toxic effects.

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