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Effects of Dietary Antioxidant Supplementation on the Development of Malignant Lymphoma and Other Neoplastic Lesions in Mice Exposed to Proton or Iron-Ion Radiation

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Abstract

Malignancy is considered to be a particular risk associated with exposure to the types of ionizing radiation encountered during extended space flight. In the present study, two dietary preparations were evaluated for their ability to prevent carcinogenesis in CBA mice exposed to different forms of space radiation: protons and highly energetic heavy particles (HZE particles). One preparation contained a mixture of antioxidant agents. The other contained the soybean-derived Bowman-Birk protease inhibitor (BBI), used in the form of BBI Concentrate (BBIC). The major finding was that there was a reduced risk of developing malignant lymphoma in animals exposed to space radiation and maintained on diets containing the antioxidant formulation or BBIC compared to the irradiated animals maintained on the control diet. In addition, the two different dietary countermeasures also reduced the yields of a variety of different rare tumor types observed in the animals exposed to space radiation. These results suggest that dietary supplements could be useful in the prevention of malignancies and other neoplastic lesions developing from exposure to space radiation.

INTRODUCTION

The adverse biological effect of most concern after exposure to the types of radiation encountered during space travel is the development of malignancy. Highly energetic heavy charged particles known as HZE particles and protons are among the most biologically significant components of space radiation. While there are many different types of cellular and molecular damage induced by HZE particles and protons, our studies have shown that these types of ionizing radiations can induce oxidative stress in cells (1–3) and animals (4–6). Oxidative stress (7) is a term that describes the biological damage of DNA, lipids and proteins by either oxygen reactive organic radicals or oxygen radicals, e.g., the hydroxyl radical. The hydroxyl radical is thought to be the primary free radical leading to the known adverse biological effects of radiation (8). Oxidative stress results whenever there is an imbalance between the pro-oxidants and antioxidants, favoring the pro-oxidants. Ionizing radiation is a pro-oxidant. Because the levels of oxidative stress are expected to be higher than normal during space travel due to the higher doses of radiation to which astronauts are exposed, the use of antioxidants could conceivably counteract the effects of radiation-induced oxidative stress in astronauts during space flight, thereby preventing the

downstream effects of the excessive oxidative stress induced by radiation, such as the development of malignancy.

It is clear from animal studies that deficiencies of certain vitamins and minerals can play major roles in carcinogenesis (9, 10), whereas adequate dietary concentrations of several different vitamins and minerals reduce the risk of cancer (9). Human intervention studies have shown that vitamin and mineral supplements can have a major preventive effect on carcinogenesis in populations that are nutritionally deficient for these dietary factors [e.g., refs. (11, 12)]. With the oxidative stress associated with space travel, it is conceivable that astronauts may deplete their vitamins and mineral stores more rapidly than other individuals and may need considerably higher amounts of antioxidant vitamins/minerals than the established Recommended Dietary Allowances (RDAs). There is some evidence that astronauts do deplete their vitamins during space flight, because there is a decrease of bio-reduction capacity [measured as plasma total antioxidant status (TAS)] in astronauts after long-duration space flights (13). Thus supplementation with antioxidant vitamins is likely to reduce the radiation-induced damage associated with space travel.

It was previously believed that the induction of malignancy by HZE particles, representing a form of high-linear energy transfer (LET) radiation, might not be a modifiable phenomenon. However, recent research has indicated that HZE-particle-induced skin carcinogenesis in rats can be suppressed by retinyl acetate (14). The challenge to researchers in this field now is to find agents that can prevent malignant transformation induced by exposure to HZE particles and other types of space radiation in multiple organ or tissue sites without significant toxicities or adverse side effects. The present study was undertaken to evaluate two different antioxidant dietary supplements, an antioxidant supplement containing a mixture of antioxidant agents and Bowman Birk Inhibitor Concentrate (BBIC), as potential countermeasures against space radiation-induced malignancy.

A number of nutritional supplement compounds that were expected to have antioxidant properties were evaluated for their abilities to affect space radiation-induced oxidative stress. L-Selenomethionine (SeM), N-acetyl cysteine (NAC), ascorbic acid, co-enzyme Q10, α -lipoic acid and vitamin E succinate were observed to have significant suppressive effects on space radiation-induced oxidative stress *in vitro* both when they were evaluated as single agents and when they were evaluated as a combination of agents (2). It has been demonstrated that this combination of antioxidant compounds is also highly effective in protecting against HZE-particle- and proton-induced cytotoxicity (15), space radiation-induced transformation in cultured human thyroid epithelial cells (3, 15), and radiation-induced oxidative stress in mice and rats exposed to radiation from HZE particles, protons and γ rays (4–6). None of these agents alone can completely prevent or suppress these biological phenomena, but when used together as a combination, the agents work together to have a highly significant suppressive effect on adverse biological effects of space radiation.

The soybean-derived protease inhibitor known as the Bowman-Birk inhibitor (BBI) was also studied for its ability to affect radiation-induced malignancies in this animal study. BBI was evaluated as BBI Concentrate (BBIC), which is the form of BBI used in human trials of BBI, as reviewed elsewhere (16–18). BBIC is an Investigational New Drug (IND), and numerous human trials with this anticarcinogenic agent have already been performed, as recently reviewed (18). BBIC has been evaluated extensively in rodent toxicity studies [e.g., refs. (19–21)] as part of its evaluation as an IND agent; it is known to be non-toxic, even at levels considerably higher than the dietary concentration used in this study, in both rats and mice.

The antioxidants and BBIC evaluated in this study have long histories of safe use in human populations; thus they could conceivably be useful without significant toxicities or adverse side effects during space travel as countermeasures for adverse space radiation biological effects.

MATERIALS AND METHODS

Antioxidants and Bowman Birk Inhibitor Concentrate

L-Selenomethionine (SeM), ascorbic acid, *N*-acetyl cysteine (NAC), α -lipoic acid, vitamin E succinate, and co-enzyme Q10 were purchased from Sigma Chemical Company (St. Louis, MO). Bowman Birk Inhibitor Concentrate (BBIC) was produced by Central Soya Co., Inc. (Ft. Wayne, IN).

Animal Food Preparation

The AIN-93G rodent diet, which is commonly used for NCI-funded cancer chemoprevention studies in animals, was used as the control diet and the base diet for preparation of the antioxidant- and BBIC-supplemented diets. The AIN-93G diet was purchased from Bio-Serv (Frenchtown, NJ) and is a defined diet prepared according to good laboratory practice guidelines, with or without the supplements. The antioxidant and BBIC supplements were provided to Bio-Serv for inclusion in the diets. The food was prepared by Bio-Serv at approximately 3-month intervals, a period short enough to avoid depletion of vitamins by air oxidation. The antioxidant-supplemented diet contains SeM (0.06 mg/kg diet), NAC (171.4 mg/kg diet), α -lipoic acid (85.7 mg/kg diet), vitamin E succinate (71.4 mg/kg diet), co-enzyme Q10 (27.9 mg/kg diet), and ascorbic acid (142.8 mg/kg diet). The levels of SeM, vitamin E and ascorbic acid used in this study are equivalent on a weight basis in humans to the established maximum level of daily nutrient intake that is likely to pose no risk of adverse effects. For NAC, co-enzyme Q10 and α -lipoic acid, for which the maximum levels of daily nutrient intake have not been established, the concentrations have been chosen based on the highest doses used in previous human studies that showed no apparent chronic toxicity [at concentrations up to 2400 mg/day of NAC (22), 390 mg/day of co-enzyme Q10 (NCI Cancer Information at www.cancer.gov/cancer_information), and 1200 mg/day of α -lipoic acid (23)]. The BBIC-supplemented diet contains 1% (w/w) BBIC.

Animal Care and Radiation Treatment

Male CBA/JCR HSD mice 7–8 weeks of age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice used for a short-term pilot study were housed in a University of Pennsylvania (Penn) animal facility, whereas the mice used for the long-term study were initially delivered to the Brookhaven National Laboratory (BNL) animal facility for radiation treatment and were then transferred to the Penn animal facility. In both facilities, the animals were provided with standard care and free access to water and food. For the short-term pilot study at Penn, the animals were acclimated to the new environment at Penn for approximately 1 week and were then randomly assigned to treatment groups, weighed and placed on the three experimental diets for 30 days. The animals were observed twice daily for any signs of toxicity. At the end of the 30-day period, the animals were weighed and killed humanely. For the long-term animal studies, the animals were acclimated to the new environment at BNL for approximately 1 week and were then assigned randomly to one of nine treatment groups. They were placed on the experimental diets 3 days prior to the radiation exposures. Initially, there were 60 animals in all treatment groups except the iron-ion radiation group and the group receiving iron-ion radiation and the BBIC diet, which each had 72 animals initially.

Experiments using beams of iron ions (1 GeV/nucleon, approximate LET 150 keV/μm) or protons (1 GeV/nucleon, approximate LET 0.24 keV/μm) were performed at the NASA Space Radiation Laboratory (NSRL) at BNL. The animals were irradiated with 0.5 Gy iron ions (at 1 Gy/min) or 3 Gy protons (at 20 cGy/min), and sham-irradiated animals were included as controls according to the study design. The iron-ion and proton irradiations were carried out with animals irradiated in the non-stopping region (Bragg plateau) of the curve for energy deposition as a function of depth. The mice were placed into restraining devices for insertion into the beam lines approximately 3 min before irradiation. After the radiation exposure, the animals were returned to cages and fed the control diet or diets supplemented with antioxidants or BBIC according to the study design. All animals at BNL were transferred to the Penn animal facilities within a short time after the radiation exposures. At Penn, they were maintained in a University Laboratory Animal Resources (ULAR) quarantine facility for 6 weeks and were then transferred to the Penn ULAR standard animal housing facilities in which animals are maintained at 25°C with a 12:12-h light-dark cycle. The animals were evaluated twice daily for approximately 2 years for any signs of toxicity or distress, so that any moribund animals or animals in distress could be euthanized. According to the study design, any surviving animals at 2 years postirradiation were weighed and then killed.

The animal use and the procedures for the animal care and treatment were approved by the Institutional Animal Care and Use Committees (IACUCs) of the University of Pennsylvania and BNL.

Necropsy

Each animal that died during the course of the study was subjected to necropsy as near to the time of death as possible. Mice that survived until the end of the 2-year study period were necropsied after the scheduled killing. For all animals used for histopathological analyses, tissues were taken from the following sites: (a) liver: sections from left and median lobes; (b) kidney: left kidney: cross section; right kidney: longitudinal; (c) spleen: along with adjacent pancreas; (d) sternum: for bone and bone marrow, along with associated muscle which is often involved with the neoplasm; and (e) any obviously abnormal tissue(s).

The tissues were fixed in formalin using standard procedures, processed into paraffin blocks, cut at 4-μm thickness, mounted on glass slides, and stained with appropriate stains (either hematoxylin and eosin or Wright Giemsa) for histopathological examination. Special treatment (decalcification) was required for the sternum.

Histopathological Analysis

Selected criteria from recently published recommendations provided by The Mouse Models of Human Cancer Consortium established by the National Cancer Institute (24) were consulted in the evaluation of the nonlymphoid (myeloid) leukemia and other lesions derived from cells of hematopoietic origin in the mice. An additional publication that has been very helpful in developing the histopathological aspects of this study is that of Major (25), which provides detailed information about the acute myeloid leukemia induced in the strain of mouse (CBA) used in this study.

All slides were evaluated without knowledge of the treatment groups using standardized terminology, generally those terms recommended in the NCI Consortium publication (24).

Statistical Analysis

The Kaplan-Meier curves for survival were compared using a log-rank test. The fractions of animals with premalignant and/or malignant lesions were compared by a Fisher's exact test

or a χ^2 test when appropriate. The animal weights were compared by a t test. The statistical analyses were performed using Prism Version 2.01 statistical software (GraphPad Software, San Diego, CA). A P value of <0.05 was accepted as being statistically significant.

RESULTS

Pilot Study

To determine whether CBA mice maintained on the supplemented diets had any signs of toxicity, a pilot study was performed using 10 mice per group. The mice were maintained on either the control diet (AIN-93G), the control diet supplemented with the antioxidant combination, or the control diet supplemented with BBIC (with the concentrations of each agent as described below for the long-term animal study). The animals were observed for any signs of toxicity, including lack of grooming, ataxia (poor balance), abnormal gait or walking, limping, abnormal posture, paralysis, lethargic and/or inactive, weakness, anorexia, hyperactivity, tremors, twitching, hunched, convulsion, circling, labored respiration, blood in cage and/or on animal, bleeding, rough or stained coat, scratching/skin irritation, abnormal color or soft stool, diarrhea, sneezing, eye lesions, sores/wounds, discharge, distended abdomen, hernia, mass/swelling/lump, red eyes (or red tears), or excretion around nose. Mice were weighed at the beginning and end of the 30-day period on the diets. No evidence of toxicity was observed at any point during this pilot study. At the end of the 1-month observation period, the mean body weights of the control group (25.4 ± 1.7 g) and the antioxidant treatment group (25.8 ± 1.2 g) were not significantly different ($P > 0.5$), whereas the mean body weight of the BBIC treatment group (27 ± 1.2 g) was approximately 6% higher than that of the control group ($P = 0.02$).

Long-Term Studies

The mice were carefully monitored for potential toxicity of the radiation or the dietary supplements used in the study. Beginning at approximately 6 months after initiation of the experiment, 12 mice demonstrated a syndrome in which they rotated their heads along the spinal axis and walked in circles. This behavior did not seem to be associated with either the diet or treatment with radiation, and the fraction of animals with this behavior (which ranged from 0% to 6% in the nine treatment groups) did not differ between the control group and any of the other eight treatment groups ($P = 0.20$ by Fisher's exact test). No other abnormalities were observed in these animals with this behavior at the time when the behavior was observed. These mice were maintained as part of their assigned treatment group until they died or were killed at the end of the experimental period.

Mice that survived to the end of the 2-year experimental period with no grossly observable tumor mass were weighed immediately prior to killing. The experimental groups were compared to appropriate control groups by a t test to determine the effects of radiation or diet supplement on the final body weights. No statistically significant effect was observed in any of the comparisons made ($P = 0.1$, Table 1). The lack of significant differences suggests that neither the antioxidant formulation nor BBIC was noticeably toxic to the animals in this long-term study.

The effect of the proton and iron-ion radiation on mouse survival was determined by comparing the survival curves of the mice fed the AIN-93G control diet and exposed to radiation (Fig. 1). Both proton and iron-ion radiation accelerated the death of mice. The survival of mice was significantly worse in mice exposed to 3 Gy protons ($P < 0.001$) or 0.5 Gy iron ions ($P < 0.05$) than in mice receiving only the sham radiation.

The effects of the antioxidant and BBIC supplementation on the survival of mice exposed to proton and iron-ion radiation were determined by comparing the survival curves of mice fed

diets with or without antioxidant or BBIC supplementation and exposed to proton (Fig. 2) or iron-ion (Fig. 3) radiation. The results show no significant differences among the different diet groups of animals exposed to either protons ($P = 0.2$) or iron ions ($P = 0.7$). Thus the supplementation of the diet with antioxidants or BBIC did not prevent the deleterious effects of proton and iron-ion radiation on survival.

The effects of radiation exposure and antioxidant and BBIC supplementation on the histopathological alterations and lesions derived from hematopoietic cells were determined by comparing the incidence of the major histopathological alterations and lesions arising from cells of hematopoietic origin among different treatment groups (Table 2). The effective number of animals in each treatment group was used to calculate the fraction of animals with lesions, and it does not include those animals that were lost during the study and were not available for necropsy (e.g., due to cannibalism, severe autolysis, etc.). All animals included in the effective number of animals died at 200 or more days postirradiation and were considered to be at risk for the development of premalignant and malignant lesions. The results demonstrate that 51% to 65% of the animals in the various treatment groups had hepatocellular adenoma or carcinoma, and none of the experimental factors (i.e., proton or iron-ion radiation with or without antioxidant or BBIC dietary supplementation) had a significant effect on the fraction of animals having hepatocellular adenoma or carcinoma ($P > 0.2$).

Malignant lymphoma was observed less frequently than hepatocellular adenoma or carcinoma in this study. A total of 54 malignant lymphomas were observed. Malignant lymphoma cells were observed in the spleen in almost all of the animals having malignant lymphoma. Malignant lymphoma lesions were also found in the liver (10 mice), lung (1 mouse), sternal muscle (2 mice), kidney (3 mice), lymph node (1 mouse), diaphragm (1 mouse), heart (3 mice) and sternal bone marrow (2 mice). Exposure to proton radiation at a dose of 3 Gy significantly increased the fraction of mice with malignant lymphoma from 0.06 (Group 1: Control) to 0.27 (Group 4) (Table 2 and Fig. 4A, $P < 0.01$). For mice fed the antioxidant- or BBIC-supplemented diet and exposed to 3 Gy proton radiation, the fractions of animals with malignant lymphoma were 0.08 (Group 5) and 0.10 (Group 6), respectively, and were not significantly higher than the control group ($P > 0.6$). Thus dietary supplementation with antioxidants and BBIC significantly protected against proton radiation-induced malignant lymphoma in this study.

Myeloid leukemia was extremely rare in the proton-irradiated animals, with only two mice, both in the proton treatment group (Group 4), developing myeloid leukemia. In both animals, myeloid leukemia cells were observed in the spleen, liver and sternal bone marrow. Myeloid leukemia cells were also observed in the kidney of one of these animals and in the heart of the other animal. Myeloid hyperplasia was observed in the spleen tissue of some of the animals in this study. Since hyperplasia is considered to be a premalignant lesion and the number of animals with myeloid leukemia was too small for a meaningful statistical analysis, the animals having myeloid leukemia or myeloid hyperplasia in the spleen were combined to determine statistically significant differences between treatment groups. Among the nine treatment groups, the fraction of animals with premalignant and malignant lesions of myeloid origin ranged from 0 to 0.13 (Table 2). The highest fraction (0.13), which was observed in the mice fed the control diet and exposed to proton radiation (Group 4), was significantly higher than the fractions observed in the sham-irradiated mice fed the control diet (Group 1, $P < 0.05$) or diets supplemented with antioxidants (Group 2, $P < 0.01$) or BBIC (Group 3, $P < 0.05$). For the mice fed the antioxidant- or BBIC-supplemented diets and exposed to 3 Gy proton radiation, the fractions of animals with premalignant and malignant lesions of myeloid origin were 0.02 (Group 5) and 0.00 (Group 6), respectively, which were not significantly higher than the control group ($P = 1.00$). These results indicate

that exposure to proton radiation at a dose of 3 Gy increased the risk of developing premalignant and malignant lesions of myeloid origin, and this risk was reduced in irradiated animals by dietary supplementation with antioxidants or BBIC.

For the mice fed the control diet, exposure to iron-ion radiation at a dose of 0.5 Gy increased the fraction of animals with malignant lymphoma from 0.06 (Group 1) to 0.19 (Group 7), and the increase was marginally significant ($P = 0.05$). For the mice fed the antioxidant- or BBIC-supplemented diets and exposed to 0.5 Gy iron-ion radiation, the fractions of animals with malignant lymphoma were 0.09 (Group 8) and 0.11 (Group 9), respectively, which were not significantly higher than the control group ($P > 0.5$). These results indicate that exposure to iron-ion radiation at a dose of 0.5 Gy increased the risk of developing malignant lymphoma, and this risk was reduced in irradiated animals by dietary supplementation with anti-oxidants or BBIC.

The fraction of animals with premalignant and malignant lesions of myeloid origin in mice fed the control diet and exposed to iron-ion radiation at a dose of 0.5 Gy was 0.08 (Group 7), which was higher than the fractions in control group (0.02, Group 1) or the iron-ion-irradiated mice fed the diets supplemented with antioxidants (0.02, Group 8) or BBIC (0.03, Group 9). However, the difference was not statistically significant ($P > 0.2$).

In addition to the premalignant and malignant lesions derived from hematopoietic cells, a number of other relatively rare premalignant or malignant lesions were observed (Table 3). The most frequent of the rare tumor types was observed grossly as a mass near the eye of the mouse. Histopathological analysis indicated that most of these masses were adenomas or adenocarcinomas arising from within the harderian glands of the mice; thus they are referred to here as harderian gland tumors. Harderian gland tumors have been reported and studied extensively in radiation carcinogenesis studies in rodents (26–28). It is noteworthy that the malignant harderian gland tumors (adenocarcinomas) were observed only in proton- or iron-ion-irradiated animals maintained on the control diet, while no malignant harderian gland tumors were observed in the proton- or iron-ion-irradiated animals maintained on the antioxidant-or BBIC-supplemented diets.

While the analysis of the prevalence of any individual rare premalignant or malignant tumor type did not result in statistically significant differences between the various treatment groups, the total number of premalignant and malignant lesions in all of the tissues examined for the study did result in some statistically significant differences between treatment groups when evaluated by a Fisher's exact test, as indicated in Table 3. Exposure to 3 Gy proton radiation increased the fraction of animals with rare malignant and premalignant lesions from 0.08 (Group 1) to 0.24 (Group 4), and the increase was statistically significant ($P < 0.03$). For the mice exposed to proton radiation and fed the antioxidant- or BBIC-supplemented diets, the fraction of animals with rare malignant and premalignant lesions was 0.02 (Group 5 and 6), which was not significantly higher than that of the control group (Group 1, $P = 0.3$). These results indicate that exposure to proton radiation at a dose of 3 Gy increased the risk of rare malignant and premalignant lesions, which were prevented by dietary supplementation with antioxidants or BBIC.

Exposure to 0.5 Gy iron-ion radiation increased the fraction of animals with non-hematopoietic malignant and pre-malignant lesions from 0.08 (Group 1) to 0.14 (Group 7); however, the increase was not statistically significant ($P > 0.3$). For the mice exposed to the iron-ion radiation and fed the antioxidant- or BBIC-supplemented diets, the fractions of animals with rare malignant and premalignant lesions were 0.06 (Group 8) and 0.05 (Group 9), respectively, which were not significantly higher than that of the control group (Group 1, $P > 0.6$).

Due to the relatively low incidence rates of malignant lymphoma and rare tumors in the individual treatment groups, the nine treatment groups described in Tables 2 and 3 were merged for statistical analyses (by a χ^2 test) of the overall effects of antioxidant or BBIC supplementation on proton- or iron-ion-induced malignant lymphoma, rare tumors, and harderian gland tumors (when considered separately from the other rare tumors). These combined treatment groups were as follows: Sham Radiation Control (Groups 1, 2 and 3), Radiation (Groups 4 and 7), and Radiation plus Antioxidants or BBIC (Groups 5, 6, 8 and 9), as indicated in Tables 2 and 3. The results show that exposure to proton or iron-ion radiation significantly increased the incidence rates of malignant lymphoma (Fig. 4B, $P < 0.001$) and rare tumors ($P < 0.02$) as well as harderian gland adenomas and adenocarcinomas ($P = 0.0003$) and dietary supplementation with antioxidants or BBIC reduced the incidence rates of malignant lymphoma and rare tumors, and harderian gland adenomas and adenocarcinomas, in the irradiated animals to levels that were not significantly higher than the Sham Radiation Control group ($P = 0.5$).

DISCUSSION

The present study evaluated the effects of dietary supplements on radiation-induced carcinogenesis in CBA mice irradiated with protons or iron ions. The results demonstrate that exposure to space radiations (proton and iron-ion radiation) significantly increases the incidence rates of malignant lymphoma and rare tumor types, which were prevented by dietary supplementation with antioxidants or BBIC. A significant increase in premalignant and malignant lesions of myeloid origin was observed in mice exposed to 3 Gy proton radiation and fed the control diet but not in mice exposed to 3 Gy proton radiation and fed a diet supplemented with antioxidants or BBIC. Thus antioxidants and BBIC are potentially effective countermeasures against space radiation-induced neoplastic alterations.

Liver tumors, both hepatocellular adenomas and carcinomas, were the most commonly observed tumor types in this study, and the incidence rates of liver tumors in CBA mice were not significantly affected by exposure to radiation with protons or iron ions or by treatment with anti-oxidants or BBIC. This was somewhat surprising, because BBIC treatment has previously been shown to suppress liver carcinogenesis in Min mice. CBA mice are known to have high spontaneous incidence rates of liver tumors (29), as are Min mice. The different results observed for BBIC treatment in CBA mice and Min mice (30) suggest that the nature of the predisposing genetic susceptibility to liver carcinogenesis in these two strains of mice is likely to be very different.

The most frequent of the rare tumor types observed in this study were harderian gland adenomas and adenocarcinomas, which were observed only in proton- or iron-ion-irradiated mice. These neoplastic lesions have been evaluated previously in rodent carcinogenesis studies [e.g., refs. (26–28)]. Exposure to proton and iron-ion radiation resulted in a statistically significant increase in the incidence rates of harderian gland tumors, and irradiated animals maintained on the antioxidant or BBIC diets had incidence rates comparable to those in control animals. Other rare tumors observed were lung tumors. CBA mice are also thought to be genetically susceptible to the induction of lung tumors (29), but very few of these lesions were observed in any of the treatment groups. The present study showed that exposure to proton and iron-ion radiation increased the incidence rates of rare neoplastic lesions in organs/tissues other than those of hematopoietic origin, and these incidence rates were decreased or prevented by the nutritional supplements evaluated in this study.

It is noteworthy that so few cases of malignant lymphoma were observed in this study, given the literature references suggesting that X rays can induce malignant lymphoma in CBA

mice [e.g., ref. (31)]. It was expected that the yield of malignant lymphoma in proton-irradiated animals would be much like that observed for X radiation, since the dose of proton radiation used in this study was the same as that used previously for X-irradiated animals, in which a statistically significant number of cases of malignant lymphoma were observed (31). The yield of malignant lymphoma from proton radiation in this study was below the yields observed for X-irradiated CBA mice. These results appear to indicate that protons may be less effective than X rays in inducing malignant lymphoma, with the relative biological effectiveness (RBE) being below 1. Some of the differences in yields of malignant lymphoma in X- and proton-irradiated CBA mice could be due to promotional factors, which were absent in the mice used in this study. It is well known that other factors have a major influence on the development of radiation-induced malignant lymphoma, such as dietary restriction (32, 33), hormones (34, 35) and exposure of the irradiated animals to turpentine (36), which is considered to be a classic promoting agent for carcinogenesis (37, 38). Diet alone is known to have major effects on carcinogenesis. It is possible that certain dietary factors missing in this study, and present in other studies, may have contributed to the low yields of proton-induced malignant lymphoma observed in this study.

In this study, 3 Gy of proton radiation resulted in considerably more neoplastic lesions arising from cells of hematopoietic origin as well as other organs/tissues than 0.5 Gy of iron-ion radiation. Differences in yields of tumors for different types of radiations are normally expressed in terms of the RBE, which is the ratio of the dose of a standard radiation, normally X rays or γ rays, to the dose of the type of radiation evaluated, to produce the same tumor yield. While RBE values cannot be calculated from the results, because X rays or γ rays were not used in these studies, the differences in yields between proton-irradiated and iron-ion-irradiated animals can be determined. If the results were exactly the same for 3 Gy proton radiation and 0.5 Gy iron-ion radiation, it would suggest that the results for a dose of 0.5 Gy iron ions were equivalent to those for a dose of radiation from protons that was six times higher. Because 0.5 Gy of iron-ion radiation resulted in lower yields of neoplastic lesions arising from cells of hematopoietic origin as well as tumors arising from cells of epithelial and connective tissue origin when compared to the results of 3 Gy proton radiation, the results suggest that iron-ion radiation is less than six times as effective as proton radiation for these end points. This is a little surprising since data obtained previously indicate that the RBE values for HZE-particle radiation compared to photon radiation are greater than 6 for carcinogenesis and related end points [e.g., refs. (26–28, 39, 40)], and the biological effects of photons are often thought to be roughly comparable to those of protons for many different biological end points (e.g. (41, 42).

The major finding from this study is that the risk of developing malignant lymphoma in mice exposed to space radiation and maintained on diets containing the antioxidant or BBIC dietary supplements was reduced to levels that were indistinguishable from the sham-radiation control. The animals with malignant lymphoma in this study rarely had bone marrow involvement; only two animals with malignant lymphoma had lymphoma cells in the bone marrow samples taken. It is assumed that the malignant lymphoma observed in this study is thymic lymphoma (a thymus-dependent T-cell malignancy), which is a neoplastic lesion that has been induced in animals by radiation exposure in numerous previous studies (43). For many years, it was thought that thymic lymphomas developed as a result of activation of the “radiation leukemia virus” that was then involved in the causation of the disease, as originally proposed by Lieberman and Kaplan (44). This interpretation of data relating to the causation of radiation-induced thymic lymphomas is no longer accepted without qualification, as reviewed by Duplan *et al.* (45). It has already been observed that the development of malignant lymphoma is influenced by non-carcinogenic secondary factors. As one example, radiation-induced lymphoma in animals can be reduced by the postirradiation administration of alpha 2-macroglobulin, a human protease inhibitor (46).

BBI/BBIC is also a protease inhibitor that appears to be capable of suppressing the developing of malignant lymphoma, as observed here. While the assumptions in this study are that a suppression of radiation-induced oxidative stress by the antioxidants and BBIC-containing diets are mechanistically involved in the prevention of malignant lymphoma, other mechanisms may also be involved.

The major conclusions of the study are that the antioxidant and BBIC dietary supplements, both of which have antioxidant activities (2, 4, 5, 47, 48), are capable of reducing the yields of neoplastic lesions derived from cells of hematopoietic origin and carcinogenesis in other tissues arising from cells of both epithelial and connective tissue origin in animals exposed to the types of radiation encountered during space travel. These results suggest that dietary supplements could be useful in the prevention of malignancies and other types of neoplastic lesions developing from exposure to space radiation.

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FIG. 1.

Survival of mice exposed to proton and iron-ion radiation. Male ICR mice aged 4–5 weeks were irradiated with 3 Gy of 1 GeV/nucleon protons or 1 GeV/nucleon iron ions at the Brookhaven National Laboratory. The animals were maintained on the AIN-93G rodent diet (Control Diet) and observed for 2 years after the radiation exposure. $P < 0.05$ for 1 GeV/nucleon iron ions compared to sham exposure; $P < 0.05$ for protons compared to sham exposure.

FIG. 2.

Survival of mice exposed to proton radiation. Male ICR mice aged 4–5 weeks were irradiated with 3 Gy of 1 GeV protons at the Brookhaven National Laboratory. The animals were maintained on the AIN-93G rodent diet with or without (Control) supplementation of anti-oxidants or BBIC and observed for 2 years after the radiation exposure. $P > 0.5$ for antioxidants compared to control diet; $P > 0.2$ for BBIC compared to control diet.

FIG. 3.

Survival of mice exposed to 1 GeV/nucleon iron-ion radiation. Male ICR mice aged 4–5 weeks were irradiated with 0.5 Gy of 1 GeV/nucleon iron ions at the Brookhaven National Laboratory. The animals were maintained on the AIN-93G rodent diet with or without (Control) supplementation of antioxidants or BBIC and observed for 2 years after the radiation exposure. $P > 0.7$ for antioxidants compared to control diet; $P > 0.7$ for BBIC compared to control diet.

FIG. 4.

Effects of antioxidant (AOX) and BBIC supplements on proton- and iron-ion radiation-induced malignant lymphomas or rare tumors. The mice were maintained on diets with or without antioxidant or BBIC supplementation and exposed to 1 GeV/nucleon iron ions (0.5 Gy) or protons (3 Gy). Sham-irradiated animals were included as controls. After the radiation exposure, the animals were returned to their cages and fed the control diet or diets supplemented with antioxidants or BBIC according to the study design and observed for approximately 2 years. The effective number of animals in each treatment group was used to calculate the fractions of animals with malignant lymphoma or rare tumors. The fractions of mice with malignant lymphomas (panel A) and/or rare tumors (panel B) were compared by Fisher's exact test (panel A) or a χ^2 test (panel B). Due to the low incidence rates of lymphoma and rare tumors in individual treatment groups, the nine treatment groups (described in Tables 2 and 3) were merged into the following treatment groups for statistical analysis by a χ^2 test (panel B): Sham Radiation (Groups 1, 2 and 3), Radiation (Groups 4 and 7), and Radiation plus BBIC or Anti-oxidant (Groups 5, 6, 8 and 9).

TABLE 1

Comparison of Body Weights of Mice in the 2-Year Long-Term Study

Treatment group	Number of mice weighed	Final body weight (g)		t-test comparisons (P value compared to Group no.)		
		Mean	Standard deviation	Diet effects	Radiation effects	
1. Control	20	24.7	2.4	N/A	N/A	
2. Antioxidants	19	23.9	2.3	>0.2 compared to no. 1	NA	
3. BBIC	11	25.7	3.7	>0.3 compared to no. 1	NA	
4. Protons	7	23.4	3.6	N/A	>0.2 compared to no. 1	
5. Protons and antioxidants	5	21.8	3.2	>0.4 compared to no. 4	>0.1 compared to no. 2	
6. Protons and BBIC	1	25	N/A (n = 1)	N/A (n = 1)	N/A (n = 1)	
7. Iron ions	19	24.4	2.9	N/A	>0.7 compared to no. 1	
8. Iron ions and antioxidants	12	24.1	3.3	>0.7 compared to no. 7	>0.8 compared to no. 2	
9. Iron ions and BBIC	11	23.7	2.1	>0.4 compared to no. 7	>0.1 compared to no. 3	

Notes. Mice that survived to the end of the 2-year experimental period with no grossly observable tumor mass were weighed immediately prior to killing. Mice with a grossly observable tumor mass were not included in the body weight analysis to avoid the body weight being skewed with tumor mass.

Major Histopathological Alterations and Neoplastic Lesions Derived from Hematopoietic Cells in Mice Irradiated with Protons and Iron Ions^a

TABLE 2

Treatment group	Number of animals with histopathological alteration/effective number of animals and comparison with Control group by Fisher's exact test				
	Hepatocellular adenoma or carcinoma	Malignant lymphoma ^a	Premalignant or malignant lesions of myeloid origin		
			Myeloid hyperplasia in spleen	Myeloid leukemia	Combined
1. Control	34/52 = 0.65	3/52 = 0.06	1/52 = 0.02	0/52 = 0.00	1/52 = 0.02
2. Antioxidants	33/53 = 0.62 (<i>P</i> > 0.8)	4/53 = 0.08 (<i>P</i> = 1)	0/53 (<i>P</i> > 0.4)	0/53 = 0.00	0/53 = 0.00 (<i>P</i> > 0.4)
3. BBIC	35/53 = 0.66 (<i>P</i> = 1)	4/53 = 0.08 (<i>P</i> = 1)	1/53 = 0.02 (<i>P</i> = 1)	0/53 = 0.00	1/53 = 0.02 (<i>P</i> = 1)
4. Protons	29/45 = 0.64 (<i>P</i> = 1)	12/45 = 0.27 (<i>P</i> < 0.01)	4/45 = 0.09 (<i>P</i> > 0.1)	2/45 = 0.04 (<i>P</i> > 0.2)	6/45 = 0.13 (<i>P</i> < 0.05)
5. Protons and antioxidants	27/48 = 0.56 (<i>P</i> > 0.4)	4/48 = 0.08 (<i>P</i> > 0.7)	1/48 = 0.02 (<i>P</i> = 1)	0/48 = 0.00	1/48 = 0.02 (<i>P</i> = 1)
6. Protons and BBIC	21/41 = 0.51 (<i>P</i> > 0.2)	4/41 = 0.10 (<i>P</i> > 0.6)	0/41 = 0.00 (<i>P</i> = 1)	0/41 = 0.00	0/41 = 0.00 (<i>P</i> = 1)
7. Iron ions	38/64 = 0.59 (<i>P</i> > 0.5)	12/64 = 0.19 (<i>P</i> = 0.05)	5/64 = 0.08 (<i>P</i> > 0.2)	0/64 = 0.00	5/64 = 0.08 (<i>P</i> > 0.2)
8. Iron ions and antioxidants	30/47 = 0.64 (<i>P</i> = 1)	4/47 = 0.09 (<i>P</i> > 0.7)	1/47 = 0.02 (<i>P</i> = 1)	0/47 = 0.00	1/47 = 0.02 (<i>P</i> = 1)
9. Iron ions and BBIC	38/66 = 0.57 (<i>P</i> > 0.4)	7/66 = 0.11 (<i>P</i> > 0.5)	2/66 = 0.03 (<i>P</i> = 1)	0/66 = 0.00	2/66 = 0.03 (<i>P</i> = 1)

^aOther abnormalities observed in the tissues of animals participating in this study (lesions in a single animal are separated by a semicolon): Group 1 (controls): liver—infarcted lobe; gross—necrotic lymphoid tissue; hemangiosarcoma; shin—keratin cyst with suppurative inflammation; keratin cyst and abscess and lipoma with calcification; lung—multiple bronchioloalveolar adenomas; lung—atalectasis; sternal muscle—leiomyosarcoma. Group 2 (antioxidant diet controls): liver—infarcted lobe; uterus—moderate angiectasis; liver—hepatoblastoma; lymph node—keratin cyst; spleen—malignant fibrous histiocytoma and hematocyst and gross lesion—site undetermined—histiocytic sarcoma; pancreas—cyst, mass calcified fibrotic. Group 3 (BBIC diet controls): lung—papillary bronchioloalveolar adenocarcinoma; liver—abscess; liver—hepatoblastoma; liver—basophilic foci of alteration; kidney—pyelonephritis, bilateral; liver—choleangionoma; lung—atalectasis; liver—infarcted; liver—infarcted, necrotic lobe; suppurative peritonitis—kidney; kidney, spleen—amyloidosis; gross—mass, calcified, fibrotic; spleen—lipidosis. Group 4 (Protons): lymph node—keratin cyst; cholangionoma (liver); adrenal gland—pheochromocytoma; keratin cyst; kidney—renal tubular adenoma (unilateral); kidney—moderate multifocal embolic nephritis; kidney—pyelitis (bilateral); lung—bronchioloalveolar adenoma; liver—abscess and sternal muscle—suppurative myositis; gross lesions—keratin cyst, cystic lymph node; liver—choleangiocarcinoma; kidney—metastatic pericapsular adenocarcinoma; kidney, spleen—amyloidosis and skin—keratin cyst; lung—bronchioloalveolar adenoma; Harderian gland tumors (observed grossly as masses near the eyes): adenocarcinoma; adenocarcinoma; adenocarcinoma; adenocarcinoma. Group 5 (Protons and antioxidant diet): spleen—calcified cyst; abscess between sternal muscle and bone; sternal muscle—fibrosarcoma; liver—eosinophilic focus; masses near eye(s); abscess with bacterial colonies; adenitis with cysts. Group 6 (Protons and BBIC diet): multiple hepatic abscesses; fibrosarcoma; spleen—amyloidosis; mass near eye: adenitis, dermatitis, myositis, abscess. Group 7 (Iron Ions): spleen—amyloidosis; kidney—mild pericapsular fibrosis and suppurative inflammation; gross lesion—lipoma; lung—atalectasis; lung—solid bronchioloalveolar adenoma; liver—severe congestion (infarction); kidney—severe multifocal bacterial pyelonephritis; liver—hemangiosarcoma; cyst—keratin; sternal muscle—rhabdomyosarcoma; Harderian gland tumors (observed grossly as masses near the eyes): adenoma; adenoma; adenoma; adenocarcinoma; adenocarcinoma. Group 8 (Iron ions and antioxidant diet): kidney—renal tubular cell carcinoma; small intestine—adenocarcinoma with desmoplasia; infarction of liver lobe; metastatic adenocarcinoma with abscess and hemangionoma (subcutis); kidney—mild focal nephritis; sternal muscle—suppurative peritonitis and kidney—peritonitis; kidney—multifocal tubular cell hyperplasia composed of oxyphilic cells. Group 9 (Iron ions and BBIC diet): kidney—severe chronic suppurative pyelonephritis (unilateral); spleen—severe suppurative splenitis (Corynebacterium kitchneri); sternal muscle—suppurative serositis; liver—1 section severely congested, suggesting an infarction; lung—atalectasis; heart—atrial thrombosis; kidney—pericapsular rhabdomyosarcoma; sternal muscle—myositis; sternal muscle—mild suppurative peritonitis; kidney—renal tubule carcinoma; spleen—amyloidosis; fibrosarcoma; spleen—amyloidosis.

^aThe results for the fractions of animals with malignant lymphoma for the various treatment groups were merged as follows for further statistical analyses: (1) spontaneous lymphoma development = Groups 1 (3/52) + 2 (4/53) + 3 (4/53) = 11/158. (2) space radiation exposure – protons and iron ions = groups 4 (12/64) + 7 (12/64) = 24/128. (3) space radiation exposure with countermeasures (antioxidants or BBIC) = Groups 5 (4/48) + 6 (4/41) + 8 (4/47) + 9 (7/66) = 19/202. The statistical analyses for these merged treatment groups gave the following results: Merged Groups (1) compared to (2), *P* = 0.0007; Merged Groups (1) compared to (3), *P* = 0.5, and Merged Groups (2) compared to (3), *P* = 0.004.

TABLE 3

Rare Neoplastic Alterations Observed in the Long-Term Animal Study

Treatment group	Number of mice with histopathological alterations/effective number of mice in treatment group and comparison with Control group by Fisher's exact test			
	Benign lesions		Malignant lesions	
	Histopathology	Fraction	Histopathology	Fraction
1. Control	Lipoma with calcification	2/52 = 0.04	Hemangiosarcoma	2/52 = 0.04
	Lung—multiple bronchioloalveolar adenomas		Sternal muscle—leiomyosarcoma	
2. Antioxidants	Liver—hepatoblastoma	2/53 = 0.04 (<i>P</i> = 1)	Spleen—malignant fibrous histiocytoma and histiocytic sarcoma	1/53 = 0.02 (<i>P</i> > 0.6, one mouse had 2 lesions)
	Pancreas— <i>islet</i> cell adenoma			
3. BBIC	Liver—hepatoblastoma	2/53 = 0.04 (<i>P</i> = 1)	Lung—papillary bronchioloalveolar adenocarcinoma	1/53 = 0.02 (<i>P</i> > 0.6)
	Liver—cholangioma			
4. Protons	Lung—bronchioloalveolar adenoma, two mice	5/45 = 0.11 (<i>P</i> > 0.2)	Kidney—metastatic pericapsular adenocarcinoma	6/45 = 0.13 ^b (<i>P</i> > 0.1)
	Liver—cholangioma		Liver—cholangiocarcinoma	
	Adrenal gland—pheochromocytoma		Harderian gland tumors—adenocarcinoma—4 mice ^c	
	Kidney—renal tubular adenoma (unilateral)			
5. Protons and antioxidants	None	0/48 = 0.00 (<i>P</i> > 0.4)	Sternal muscle—fibrosarcoma	1/48 = 0.02 (<i>P</i> = 1)
6. Protons and BBIC	None	0/41 = 0.00 (<i>P</i> > 0.5)	Fibrosarcoma	1/41 = 0.02 (<i>P</i> = 1)
7. Iron ions	Gross lesion—lipoma	4/64 = 0.06 (<i>P</i> > 0.6)	Liver—hemangiosarcoma	5/64 = 0.08 (<i>P</i> > 0.4)
	Lung—solid bronchioloalveolar adenoma		Sternal muscle—rhabdomyosarcoma	
	Harderian gland tumors—adenomas—2 mice ^c		Harderian gland tumors—adenocarcinomas—3 mice ^c	
8. Iron ions and antioxidants	Hemangioma (subcutis)	1/47 = 0.02 (<i>P</i> = 1)	Kidney—renal tubular cell carcinoma	3/47 = 0.06 ^d (<i>P</i> > 0.6)
			Small intestine—adenocarcinoma with desmoplasia	
			Metastatic adenocarcinoma with abscess	
9. Iron ions and BBIC	None	0/66 = 0.00 (<i>P</i> > 0.1)	Kidney—pericapsular rhabdomyosarcoma	3/66 = 0.05 (<i>P</i> = 1)
			Kidney—renal tubule carcinoma	
			Fibrosarcoma	

^aThe results for the fractions of animals (with Total—Benign and Malignant lesions) for the various treatment groups were merged as follows for further statistical analyses: (1) spontaneous lymphoma development = Groups 1 (4/52) + 2 (3/53) + 3 (3/53) = 10/158, (2) space radiation exposure—protons and iron ions = Groups 4 (11/45) + 7 (9/64) = 20/109, (3) space radiation exposure with countermeasures (antioxidants or BBIC) = Groups 5 (1/48) + 6 (1/41) + 8 (3/47) + 9 (3/66) = 8/202. The statistical analyses for these merged treatment groups gave the following results: Merged Groups (1) compared to (2), $P=0.004$, Merged Groups (1) compared to (3), $P=0.4$, and Merged Groups (2) compared to (3), $P=0.0001$.

^bTwo metastatic lesions were counted in two mice with malignant lesions (one in Group 4 and one in Group 8). Although it is clear that there was an adenocarcinoma in each of these animals, the primary sites were not identified.

^cThe results for the fractions of animals with Harderian adenoma or adenocarcinoma for the various treatment groups were merged as follows for further statistical analyses: (1) spontaneous harderian adenoma or adenocarcinoma development = Groups 1 (0/52) + 2 (0/53) + 3 (0/53) = 0/158, (2) space radiation exposure—protons and iron ions = Groups 4 (4/45) + 7 (5/64) = 9/109, (3) space radiation exposure with countermeasures (antioxidants or BBIC) = Groups 5 (0/48) + 6 (0/41) + 8 (0/47) + 9 (0/66) = 0/202. The statistical analyses for these merged treatment groups gave the following results: Merged Groups (1) compared to (2), $P=0.0003$; Merged Groups (2) compared to (3), $P=0.0001$.

^dOne animal had both a benign and a malignant tumor in this treatment group.