Effect of dietary ascorbic acid on the incidence of spontaneous mammary tumors in RIII mice


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ABSTRACT A study of the effect of different amounts of L-ascorbic acid (vitamin C), between 0.076% and 8.3%, contained in the food has been carried out with ten groups of RIII mice (seven ascorbic acid and three control groups), with 50 mice in each group. With an increase in the amount of ascorbic acid there is a highly significant decrease in the first-order rate constant for appearance of the first spontaneous mammary tumor after the lag time to detection by palpation. There is also an increase in the lag time. The mean body weight and mean food intake were not significantly different for the seven ascorbic acid groups. Striking differences were observed between the 0.076% ascorbic acid and the control groups (which synthesize the vitamin): smaller food intake, decreased lag time, and increased rate constant of appearance of the first mammary tumor. This comparison cannot be made experimentally for guinea pigs and primates because the control groups would develop scurvy.

The depletion of vitamin C (L-ascorbic acid) in cancer patients (ref. 1, pp. 120–126) and the essential role of this vitamin for the immune system (ref. 1, pp. 108–111) suggest that the dietary level of vitamin C may affect the initiation and growth of malignant tumors in man and animals. Clinical results have confirmed a beneficial effect of high vitamin C intake on patients with advanced cancer (ref. 1, pp. 129–182). Earlier studies at this institute (2, 3) showed that increased levels of dietary vitamin C delayed the onset of dermal neoplasms induced in hairless mice by ultraviolet light. Here we report on the effects of increasing dietary levels of ascorbic acid on the time to appearance, the rate of growth, and the multiplicity of spontaneous mammary tumors in RIII mice.

MATERIALS AND METHODS

Unlike humans, mice produce endogenous ascorbic acid. Nonetheless, mice were chosen as the test animals in this study because high ascorbate intake has been shown to influence the development of certain tumors in mice (2, 3). RIII/Imr virgin mice were selected because this inbred strain is characterized by transmission of mammary tumor virus (MTV) through the maternal milk to the pups (4, 5), a high spontaneous mammary tumor incidence, and a mean tumor onset age of about 12 months (6). The colony was bred at the Institute for Medical Research (Camden, NJ).

Before the main study was begun, an 11-wk pilot study was conducted, using six groups of 20 mice each. This provided experience in the use both of the certified biologically clean (CBC) facility (see below) and the procedures for collecting and recording data as well as information on the acceptance of some of the proposed diets by RIII mice.

For the main study, 864 mice, born during a 2-wk period and averaging 7 wk of age, were shipped by air freight in separate lots on four successive days. All were from the third through the sixth litters from expansion stock. Each of the mothers was at least a third-litter female. Immediately upon their receipt the few mice appearing weak or sick were removed. The others were identified (ear punching and toe clipping), transferred to individual glass jars, and transported to the CBC room, where they were housed in individual stainless steel cages.

Animal Housing and Protocol. The mice were housed in the CBC room during the entire study. This facility is designed (Class Biologically Clean, Madison, WI) to provide an environment that can be maintained at all levels of control from conventional to germ-free. The plastic-walled room with antechamber is completely self-contained, under a small positive air pressure, and supplied with sterile air (12 changes per hour). Ultraviolet-sterilized water (S. E. Group Systems Engineering, Napa, CA) was delivered automatically to each individual cage. Access to the room is through the anteroom, where the entering person dons a sealed plastic suit with its separate supply of purified air and an exhaust system. Thus the mice were isolated from direct exposure to humans. The CBC room was maintained at 22 ± 1°C and was illuminated 12 hr each day by “cool white” fluorescent lamps. Because no traffic was permitted into the room surrounding the CBC room, noise was reduced to a low level. The CBC facility was used to reduce the overall stress on the test animals to a minimum.

Food for each mouse was supplied in a hanging stainless steel feeder. Hardwood shavings were used as bedding. After initial sterilization of the CBC room by spraying with D C & R (Hess and Clark, Ashland, OH) at 1:63 in water and introduction of the animals, food was brought into the room without sterilization. Before bedding was introduced into the room it was autoclaved and sealed in double plastic bags, the outer bag being removed in the antechamber. The same procedures were used for cages, equipment, and supplies. Each cage and feeder was sanitized and the bedding was replaced on a 21-day schedule.

After a second examination of each animal 2 days after arrival, 850 mice were randomly (7) assigned to 17 groups of 50 mice each. No additional animals were housed in the CBC room for the duration of the study. Each mouse was weighed on the 7th day after arrival. On the following day the transition diet period began. This consisted of 9 days during which the diet of each group was progressively changed from

Abbreviation: CBC, certified biologically clean.
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the original standard to the assigned test diet. The end of this period was the beginning of the experiment, the mice then being 9 ± 1 wk old, with a mean (± SD) weight of 20.9 ± 1.6 g. The weight of each mouse and the food it consumed in 1 wk were thereafter determined monthly.

Mammary tumors were first observed when the mice were 31 ± 1 wk old. Thereafter each test mouse was palpated weekly and the location and diameter of each mammary tumor (measured with a caliper in the direction of the body length) were recorded. Animals were sacrificed when tumors exceeded 15 mm in diameter or became serious burdens.

Animal Health. Health profiles on 10 of the RIII virgin mice randomly selected from animals supplied for the pilot study showed all of them to be in good health, but ectoparasites (Myobius musculus) were detected on 6 of the 10 (J. D. Russell, personal communication). These mites posed no problem during the pilot study, but after the main experiment was 4 months old it became clear that the proliferation of mites could become a serious threat and some miticide treatment would be required. After discussion of the problem with several veterinarians, Atgard C (Diamond Shamrock, Cleveland, OH) was selected. This is a broad-spectrum parasiticide in the form of resin beads that contain an organophosphate (dichlorvos) as the active ingredient. After the mice were 39 wk old, a tablespoonful of Atgard C was mixed into the bedding of each cage every time the bedding was changed. To monitor the mite problem the scratch damage of each mouse was recorded each time the animal was weighed, starting at age 48 wk. Later mineral oil was therapeutically applied to severe scratch lesions and to the top of the head of each mouse as a preventive measure. These measures controlled the mites, but complete elimination was not achieved.

Diets. The food was the basic diet described by Knapka (23) with modifications1 to provide for the addition of 10% of a mixture of L-ascorbic acid, corn starch, and α-cellulose in proportions such that all diets were equicaloric.2 The basic ingredients and additives were mixed and steam-pelleted (to control Salmonella and other Gram-positive bacteria) by Simonsen Laboratory (Gilroy, CA) in commercial-scale equipment in sufficient quantities (160 kg) to provide the selected diets for the entire experiment. These diet pellets were then kept at −40°C under nitrogen until shortly before use.

Analyses of pilot lots of different diets showed a nearly complete loss of vitamin B1 (thiamin) in all diets containing added vitamin C and about 20% loss of added vitamin C. Presumably these losses occurred during the steam-pelleting. Thiamin was supplied in the drinking water by adding 20 mg/liter to ultraviolet-sterilized water in a 50-gallon (190-litter) stainless steel reservoir. Fresh thiamin solution was prepared weekly.

Analyses made before and after the main study for vitamins A and E, niacin, pyridoxine, riboflavin, and pantothenic acid in a control diet and in at least one diet with high vitamin C content showed nutritionally adequate amounts of these vitamins to be present.

Ascorbic acid contents of the diets were measured by using high-performance liquid chromatography with electrochemical detection. The mouse food was extracted with ice-cold 0.5% oxalic acid in water and the extract was filtered, diluted with 50 mM perchloric acid, and injected directly into the liquid chromatographic column (8). No vitamin C (<0.01%) was detected in the control diets. Analyses at the beginning, near the middle, and at the end of the study showed no significant change in ascorbate contents of the diets. The figures for vitamin C content are the means of five analyses done at different times before, during, and after the experiment.

Seventeen test diets were studied; we report here the appearance and growth of mammary tumors on mice fed only diets containing vitamin C as the sole test additive and on control groups. The results for the other diet groups will be the subject of a separate report. Six of the diets contained ascorbic acid at the following levels in percent by weight: 0.076, 1.86, 2.9, 4.2, 8.0, and 8.3. Two additional groups served as controls, one fed ad lib and the other receiving a "restricted" diet. Each animal in the restricted control group received 90% of the average food consumed per mouse in the test groups, with the amount being adjusted monthly. Two additional groups were used in a cross-over design. In one of these (X8.1), each mouse was on the ad lib control diet until a mammary tumor was detected, at which point that mouse was shifted to the 8.1% ascorbic acid diet. In the other cross-over group (8.1X) each mouse was on the 8.1% diet until a mammary tumor was detected, then switched to the ad lib control diet. The mice on the restricted control diet ate all the food given them. All the other groups were fed ad lib.

Diets were identified only by a color code, which was unknown to all persons involved in collecting or processing the data. Accordingly, the study was blind in the sense that none of the persons conducting the study knew which diet any particular group of mice received. The sole exception to this was the group receiving the restricted control diet. The two cross-over groups, 8.1X and X8.1, were also known to the persons feeding the mice, but they did not know their identities.

Necropsies and Histology. All of the mice, including the survivors at the end of the experiment, were subjected to necropsy. The mammary tumors, as well as selected organs, were dissected and preserved in buffered Formalin for later examination.

A section of each of the primary mammary tumors found in this study was prepared for histological examination. With few exceptions, all were mammary neoplasms of adenocarcinomatous character. Approximately one-third of the tumors were very well differentiated and noninvasive. In some classifications, these neoplasms would be regarded as benign—i.e., adenomata. The bulk of the tumors, however, were definitely malignant. Although moderately differentiated and noninvasive in appearance, these neoplasms tended to invade the adjacent tissues, including the musculature. Metastases were detected in distended pulmonary arteries, but the tumors did not penetrate the walls of the vessels. These less differentiated tumors consisted of disorganized cell groups: masses devoid of stroma and often necrotic. It should be noted that even the well-differentiated neoplasms did not show extensive stroma formation. A few tumors were uncommon variants of the mammary neoplasms: ductal, comedo-, or adenosquamous cell carcinomas. Rare cases of sarcomatous degeneration with giant cells were also present, and a few neoplasms had ulcerated through the overlying skin.

RESULTS

The data on time of appearance of the first tumor are given in Table 1, which lists the number of mice in each group that had developed the first tumor during each period of 6 weeks, the number of tumorless mice that had died from various causes during this period, and the number at risk at the beginning of each period.

Biostatistical Analysis of the Observed Age at Detection of the First Mammary Tumor. Tarone's test for trend (9) was
applied to the ages of the mice when the first mammary tumor was detected. This test is essentially the same as the method recommended by the International Agency for Research in Cancer (10), which was applied to the results obtained on the incidence of squamous cell carcinoma in irradiated hairless mice in the earlier study at this institute (3). The test was applied to the seven groups listed in Table 1 that received food containing ascorbic acid from the beginning of the experiment, viz. 0.076%, 0.86%, 2.9%, 4.2%, 8.0, and 8.3% C; also 8.1X. The group 8.1X was included because its treatment up to the time of developing the first tumor was the same as that of the other vitamin C groups except for the amount of the vitamin. Applying this test to the data shows that increasing the amount of ascorbic acid added to the diet increases the age at which the first mammary tumor appears in these mice. The statistical significance of this conclusion is extremely high, the value of P being 0.00000005.

Semilogarithmic Kaplan–Meier survival plots (11) based on Table 1 are shown in Fig. 1. The value of the semilogarithmic representation lies in the fact, pointed out by Jones (12) and by Burch (13), that for many populations of cancer patients these curves are straight lines, with the constant slope indicating a constant rate of death (in Fig. 1 a constant rate of incidence of the first tumor). The points in Fig. 1 correspond to the midpoints of the horizontal and vertical line segments in the usual Kaplan–Meier staircase diagrams. The top curve is for the groups 8.0% C, 8.1X, and 8.3% C. They have been combined into a single group, initially 150 mice, because the amount of vitamin in the diets, 8.0 to 8.3%, is nearly the same for the three groups.

The linearity of such a semilogarithmic curve, corresponding to a first-order process,§ indicates that a single additional oncogenic factor, operating by chance, is required as the last step in the progression of the cancer. This factor is affected by the amount of vitamin C in the diet, the rate constants for the five curves for 0.076%, 1.86%, 2.9%, 4.2%, and 8.1% C being 0.0212, 0.0128, 0.0149, 0.0110, and 0.0089 wk−1, respectively. The corresponding median ages at appearance of the first mammary tumor were 69.5, 92.4, 89.1, 109.9, and 124.9 wk. They are an approximately linear function of the amount of vitamin C in the food, as shown in Fig. 2.

The horizontal intercepts of the five curves (the lag times) are at ages 36.7, 38.1, 42.5, 46.8, and 47.1 wk, indicating that the lag time to observation by palpation is delayed by an increase in dietary ascorbic acid.

**Body Weight, Food Consumption, and Mite Infestation.**

Mean body weight, mean weekly food consumption, and mean severity of mite infestation for each group are given in Table 2. The mean body weights show no significant dependence on ascorbic acid content. The mean food intake drops sharply from the ad lib control of 0.076% C and then gradually to 8.0–8.3% C. The severity of skin mite lesions shows no significant dependence on vitamin C content.

**Dietary Ascorbic Acid Effect in Mice.** In our study of

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§Such a process is described by the equation \( S = S_0 \exp\left(-k(t-t_0)\right) \). In our case, \( S \) is the fraction of the mice that remain mammary-tumor-free at time \( t \), \( S_0 \) is unity (100%) at the lag time \( t_0 \), and \( k \) is the rate constant. The median age, \( t_{\text{50}} \), at which \( S = 0.5 \) (or 50%) is \( t_0 + 0.693/k \).
squamious cell carcinoma in hairless mice (3) there was an indication that the rate of incidence of the skin lesions was increased by 0.3% ascorbic acid in the food. Using the means of the recorded time of incidence for the first tumors 2, 4, 6, and 10 mm in diameter (table 3 of reference 3) the values of

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\begin{align*}
W & = 23.8 \pm 1.7 \\
F & = 36.0 \pm 7.5 \\
M & = 15
\end{align*}
\]

The rate constant \(k\) were 0.042 and 0.043 wk\(^{-1}\) for 0% C and 0.047 and 0.046 wk\(^{-1}\) for 0.3% C. The values of \(k\) found in the present study, 0.0164 wk\(^{-1}\) for 0% C (alc + X8.1) and 0.0212 wk\(^{-1}\) for 0.076% C, show a still larger effect (Fig. 3). In addition, the intercept decreases from 40.1 to 36.7 wk. There is, moreover, a pronounced effect on food intake, which is decreased by 9.6%. This decrease may be the result of the acidity of the food with 0.076% ascorbic acid, but the 0.076% C mice do not show the decrease in body weight observed for the group with restricted food intake. It is not possible to study such an effect with guinea pigs or human beings, because there can be no control groups with zero intake of vitamin C since they would die of scurvy. We emphasize that this effect found in mice in this and the earlier study (3) at the lowest level of added dietary vitamin C cannot be related to humans. We think that this effect in mice involves an
Interaction with their mechanism for synthesizing ascorbate in the liver cells.

**Effect of Restricting Food Intake.** The values of \( k \) for the ad lib and the restricted control groups differ somewhat (Fig. 3), being 0.0164 and 0.0187 wk\(^{-1}\), respectively. The lag times for these groups are 40.1 and 50.4 wk and the median ages for tumor appearance are 82.5 and 87.3 wk, respectively, with the larger time referring to the restricted food intake group in both cases. Decreased food intake does not explain the decrease in \( k \) observed for the high-vitamin-C groups.

**Rate of Growth of Tumors.** Analysis of the weekly data on the diameter of each tumor from the time of its detection indicated no significant correlation between the rate of growth and the ascorbic acid content of the diet. The mean diameter of the tumors at detection was 7 mm. The mean growth of single tumors was 3.1 mm the first week, 2.6 the second, 1.5 the third, and 1.9 the fourth, with large variations from these means. The average time for single tumors to attain a diameter of 15 mm was approximately 4 wk, again with wide variations from the mean. Most of the tumors grew steadily from the time of detection, but some stabilized and fluctuated in size for several weeks before growing larger.

In many cases more than one mammary tumor developed on a single animal. The incidence of these multiple tumors is shown in Table 3. The time between detection of the first and second tumors averaged 3.9 wk, with a range of 0 to 10 wk. The rate of growth of the first tumor appeared to be lessened by the presence of a second tumor; there was a mean time of 5 wk from detection to a diameter of 15 mm. In the 18 cases in which the second tumor attained a diameter of 15 mm, the mean time was about the same for this growth as it was for single tumors. Analysis of the data of Table 3 by use of the Jonckheere and Terpестra nonparametric test for trend (14) shows that increasing doses of vitamin C significantly decrease the number of tumors per mouse (\( P = 0.000008 \)).

**Effect of Changing Diet After Detection of the First Mammary Tumor.** The potential effects of changing the diet after a first tumor has been detected are changes in the subsequent rate of growth of tumors and in the number of additional tumors that may develop. No significant effect of changing the diet was observed for either of these quantities for the mice on either the X8.1 or the 8.1X schedule.

**DISCUSSION**

The absence of carcinogenicity for high levels of vitamin C in the diets of rodents was recently confirmed by Douglas et al. (15). Although other studies have reported the beneficial effects of vitamin C in murine cancer, apparently none has employed spontaneous mammary tumors. In studies by others, ascorbate reduced the growth rate, delayed the onset, or prolonged the life span of mice bearing the tumors. The origin of the tumors varied: ovarian tumor transplants beneath the subrenal capsule (16), sarcoma-180 subcutaneous implants (17), Ehrlich tumor cells injected intramuscularly (18, 19), melanoma cells injected subcutaneously (20, 21), mammary adenocarcinoma transplanted subcutaneously (22), and squamous cell carcinomas induced by ultraviolet irradiation (2, 3). The main result of this study, which involved ten groups, each of 50 RII mice, a strain that develops spontaneous mammary tumors, is that the rate of appearance of the first palpable tumor decreases significantly with an increase in the amount of ascorbic acid in the food, with the median age of appearance increasing as an approximately linear function of the amount. The conclusion that increased intake of ascorbic acid decreases the rate of tumor appearance has extremely high statistical significance (\( P = 0.0000005 \)).

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