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Effects of intake of L-ascorbic acid on the incidence of dermal neoplasms induced in mice by ultraviolet light

(cancer/skin/vitamin C)

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ABSTRACT **We have carried out a study of large malignant skin tumors (squamous cell carcinomas) and other lesions in hairless mice (groups of 38–45) intermittently exposed to ultraviolet light over a period of 15 weeks, beginning when the mice were about 10 weeks old. The several groups were given a standard diet with 0%, 0.3%, 5%, and 10% added L-ascorbic acid (vitamin C) throughout the study. No lesions developed in unirradiated control groups. The lesions were counted every 14 days for 4 months, beginning 4 weeks before the end of the period of irradiation. The observed incidence of lesions of several sizes during successive time periods was analyzed by the statistical method recommended by a committee of the International Agency for Research on Cancer. A pronounced effect of vitamin C in decreasing the incidence and delaying the onset of the malignant lesions was observed with high statistical significance.**

Interest in vitamin C and other dietary factors in relation to the incidence of and mortality from cancer in human beings has been stimulated by the studies of the value of vitamin C for patients with advanced cancer in Vale of Leven Hospital (Loch Lomondside, Scotland) from 1971 on (1–9). In 1973 it was suggested to one of us (L.P.) that we carry on studies of vitamin C in relation to cancer in animals (8). We began these studies in 1976, and we recently published a report on four studies of vitamin C and vitamin E in relation to skin cancer in hairless mice exposed to ultraviolet light in a standard way (10). In the present paper we report on another study on irradiated mice.

About 10 years ago, Homer Black and his collaborators reported that cholesterol-5*d*,6*d*-epoxide (cholestan-5*d*,6*d*-epoxy-3-ol), a carcinogenic sterol, is formed in skin on ultraviolet irradiation, both in humans (11) and in mice (12, 13). They also reported that dietary anti-oxidants [a mixture of L-ascorbic acid, butylated hydroxytoluene, D,L-α-tocopheryl acetate (vitamin E), and glutathione] suppressed tumor formation induced in hairless mice by exposure to ultraviolet light (12). Black has given us much information and advice about his techniques.

MATERIALS AND METHODS

The hairless mouse is a mutant that loses its hair about 3 weeks after birth, providing easy visibility of skin lesions. It does not have the thymus defect of the *nude* mutant. Our mice, all females, were of the SK *h-hr* strain bred at Temple University Health Sciences Center (Philadelphia). They were obtained in March and April of 1980 at age 6–9 weeks. They were maintained for at least 2 weeks on Purina certified rodent chow no. 5002 to stabilize the colony before the special diets were begun. They were then distributed five mice to a cage so that the combined weight and mean age per cage were standardized (individual weights, 17.6–32.9 g; ages, 8–11 weeks). Irradiation was begun 10 days later and was continued 5 days per week for about 15 weeks to a total exposure of 135 J/cm^2. The radiation was from GE-UA3 mercury arc lamps as described by Black except that an automatic timing device was added. The daily exposure began at 1.13 J/cm^2 and was increased every 2 weeks to a final value of 1.97 J/cm^2 to compensate for epidermal thickening. The mice were observed through a window during the irradiation and it was verified that they did not huddle or pile on top of one another; each mouse received a full dorsal exposure.

The diets are described in Table 1. They were based on Purina certified rodent chow no. 5002 purchased from Dean's Animal Feed (Belmont, CA). Dry Purina chow mash in 4-kg batches was mixed with the diet supplements and blended for about 5 min in a Patterson–Kelley twin shell blender, model LB-S-8. Deionized water was added in predetermined amounts for each diet, sufficient to moisten, and the blending was continued for 5 min. The food was then pelleted in a California pellet mill, type 3, with a 1/2-inch die, and the pellets were fan-dried at room temperature, placed in 1-gallon jars, sealed, and immediately frozen at −40°C until needed. The food was warmed to room temperature as needed and placed in the cages to be eaten ad lib. The L-ascorbic acid was purchased from Bronson Pharmaceuticals (La Canada, CA). The intake of food, measured over a 3-week period, was the same for different groups to within 6%.

RESULTS

Nature of the Lesions. In the earlier study (10), 60 of the lesions were subjected to histopathologic study by three pathologists, who agreed with one another and with Homer Black in classifying them as atypical squamous cell proliferations varying from early actinic keratoses to invasive, poorly differentiated, squamous cell carcinomas. The small lesions (up to 2 mm in diameter) were mostly actinic keratoses, and the larger lesions (6 mm or more) were squamous cell carcinomas, some ulcerated, occasionally with foci of highly anaplastic or undifferentiated cells, and some showing intense mitotic activity; others consisted of relatively well-differentiated keratin-producing cells. The lesions of intermediate size mainly represented either hypertrophic actinic keratoses or low-grade squamous cell carcinomas.

In the present study the gross readings of the lesions were made at 14-day intervals by the same person (R.W.) as in the earlier study. All lesions classified as papillomas or carcinomas were counted and recorded. The presence of other lesions—mainly small keratoses, areas of thickened skin, wounds, and blemishes—in small or large number was noted, but they were not included in the analysis. The location of the lesions (head,

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Table 1. Composition of diets

Designation	Composition
0.0% AA	Purina certified rodent chow no. 5002
0.3% AA	Chow with 0.3% added ascorbic acid
5% AA	Chow with 5% added ascorbic acid
10% AA	Chow with 10% added ascorbic acid

quadrants of the body) was also recorded to assist in noting the progress of individual lesions.

After the ninth reading, representative tumors present on the groups of irradiated mice fed normal diet or 10% AA were assigned randomized code numbers and examined histologically (Table 2). Most tumors 6 mm or larger in diameter were found to be epidermal carcinomas. This applied both to the large tumors that developed on mice fed normal diet and to the less-frequent large tumors on mice that received a diet containing 10% ascorbic acid.

Unirradiated Controls. Four groups, each of 20 mice, were followed for the entire period (nine observations at intervals of 2 weeks), without irradiation. These groups were those with the control diet, and this diet plus 0.3%, 5%, or 10% ascorbic acid. Altogether, 720 observations were made. No lesions were observed on any of the mice at any time. The lesions observed in the irradiated groups accordingly can be ascribed to the radiation and not to the dietary supplements or other factors.

Experimental Groups. We report on four irradiated experimental groups: with the control diet and with this diet plus 0.3%, 5%, or 10% L-ascorbic acid.

There were 45 mice in the control group and 38–40 in each of the other groups. There were three deaths from unrelated causes in the mice on the control diet and one in the group receiving 5% ascorbic acid. Correction was made for these deaths in the statistical analysis by the usual method of right censorship (14).

The mice were inspected and weighed every 14 days, beginning 4 weeks before the end of the period of irradiation, for a total of nine inspections. The lesions in each of five size categories were counted, identified by location, and classified as described above. In the tables we show, for each group of mice, the number of mice that had developed lesions of the indicated sizes during the 2-week period, the number at risk, and the number alive.

Statistical Treatment. The primary hypothesis—that increasing doses of vitamin C increase the likelihood of survival for a longer period before the appearance of skin lesions—was tested by application of the method formulated for the International Agency for Research on Cancer (IARC) (15) and recommended to us by Richard Peto (Imperial Cancer Research Fund Department of Cancer Studies, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, England). It involves the use of all of the groups of animals in the test of a carcinogenic agent or protective substance and is not based significantly on the assumption of a particular response function. In the procedure, a comparison is made of the number of at-risk animals in a group that develop a particular type of tumor with the number that would have been expected had the age-specific tumor onset rates been the same in all of the groups. The word "expected" means "expected on the basis of the average outcome among all the treatment groups of such animals in the whole experiment." If the observed number is greater than the expected number in some groups, then it must be less than the expected number in others, for the sum for all groups is exactly the total number for the whole set.

Table 2. Histological diagnosis of lesions on irradiated mice

	Normal diet		10% AA	
Diagnosis	2–6 mm	≥6 mm	>2–6 mm	≥6 mm
Epidermal carcinoma	1	6	0	3
Atypical hyperplasia	3	1	4	0
Hyperplasia	6	0	5	0
Retention cysts	2	0	2	0

If there appears to be a tendency for the observed numbers to be less than the expected numbers in the groups receiving the higher doses of the protective substance being tested, a significance level (a one-tailed P value) may be calculated that estimates the probability that a trend as big as or bigger than the observed trend would arise by chance alone.

The authors of the IARC report state that this statistical treatment reasonably may be considered to be the optimum and that it usually should supersede the separate pieces of information arising from statistical comparisons of various pairs of individual groups with each other or judgment of whether a plausible dose–response relationship appears to exist. They also point out that the P values should not be interpreted in an inflexible way. "A P-value of 0.001 from a well-run experiment is to most people convincing evidence that treatment does affect cancer-onset rates even in the entire absence of any supporting evidence, but a P-value around 0.05 should usually not be, and still less should the interpretation of P-values just above and just below 0.05 differ markedly."

Because the four groups in the present study were treated with diets containing increasing amounts of the same agent, the statistical methods were chosen to test specifically for trend. This procedure results in increased power over tests against general alternatives.

The end point considered was length of the time until the appearance of the first persistent lesion. Data were right censored when the animals died from causes unrelated to the experiment. The data were analyzed for lesions greater than 2, 4, 6, or 10 mm in diameter.

The appropriate statistical test for trend for censored survival data of this type is Tarone's (14) single degree of freedom procedure based on the framework developed by Peto and Peto (16), Mantel (17), and Breslow (18). The analysis begins by constructing a sequence of life tables for each time point at which observations were made. Censoring is reflected in the nonadditivity of cells across tables (dropouts are not counted in risk-set totals at time points subsequent to their end point).

From these tables, a vector X is constructed that contains in its ith component the total score from group i compared with all other groups. X is a random vector, because the outcome would not be expected to be precisely the same in a repeat of the trial. Hence the variance-covariance matrix S also must be computed. In the IARC model this is derived from applying the theory of the hypergeometric distribution to each of the life tables. The test statistic, which is sensitive to monotonic departures from the null hypothesis, is

$$(Z^*)^2 = (D'X)^2 / D'S^2D$$

where $'$ denotes transposition. Here, the test statistic depends upon choice of dose vector D. In our analysis, D was set simply equal to the actual dose levels used. Although other choices are possible, we have no *a priori* evidence that any other choice would be better. For any choice of D, $(Z^*)^2$ follows under the null hypothesis an asymptotic χ^2 distribution with one degree of freedom. The P values reported in Table 3 were calculated by taking the square root of $(Z^*)^2$ and using a table of one-tailed normal probabilities.

The calculations were made in the following way. Let N_i be the number of mice at risk at the beginning of the interval in

Table 3. Development of lesions of various sizes in mice

	Readings								
	1	2	3	4	5	6	7	8	9
Lesions ≥2 mm									
0.0% AA									
Number with lesion	0	0	2	1	7	4	3	1	5
Number at risk†	45	45	44	42	41	34	30	27	26
Total alive	45	45	44	44	44	44	44	44	42
0.3% AA									
Number with lesion	0	0	0	2	1	6	5	5	5
Number at risk	39	39	39	39	37	36	30	25	20
Total alive	39	39	39	39	39	39	39	39	39
5% AA									
Number with lesion	0	0	1	1	1	2	6	2	2
Number at risk	38	38	38	37	36	35	33	27	24
Total alive	38	38	38	38	38	38	38	38	37
10% AA									
Number with lesion	0	0	0	0	2	1	0	4	7
Number at risk	40	40	40	40	40	38	37	37	33
Total alive	40	40	40	40	40	40	40	40	40
Trend	0.00	0.00	−6.21	−9.51	−16.92	−29.64	−27.08	−1.52	−2.62
Trend sum	0.00	0.00	−6.21	−15.72	−32.64	−62.28	−89.36	−90.88	−93.49
Z*			0.88	1.46	1.91	2.8	3.35	3.01	2.7
P (one-tailed)			0.2	0.07	0.03	0.003	0.0005	0.001	0.003
Lesions ≥4 mm									
0.0% AA									
Number with lesion	0	0	1	1	2	2	3	2	2
Number at risk	45	45	44	43	42	40	38	35	33
Total alive	45	45	44	44	44	44	44	44	42
0.3% AA									
Number with lesion	0	0	0	0	2	2	3	3	4
Number at risk	39	39	39	39	39	37	35	32	29
Total alive	39	39	39	39	39	39	39	39	39
5% AA									
Number with lesion	0	0	1	0	1	0	1	2	3
Number at risk	38	38	38	37	37	36	36	35	32
Total alive	38	38	38	38	38	38	38	38	37
10% AA									
Number with lesions	0	0	0	0	1	0	2	2	0
Number at risk	40	40	40	40	40	39	39	37	35
Total alive	40	40	40	40	40	40	40	40	40
Trend	0.00	0.00	−2.47	−3.75	−7.06	−14.69	−9.40	−5.01	−19.99
Trend sum	0.00	0.00	−2.47	−6.23	−13.29	−27.98	−37.38	−42.39	−62.38
Z*			0.43	0.88	1.09	1.91	1.97	1.89	2.45
P (one-tailed)			0.3	0.2	0.15	0.03	0.025	0.03	0.007
Lesions ≥6 mm									
0.0% AA									
Number with lesion	0	0	0	1	2	1	5	1	0
Number at risk	45	45	44	44	43	41	40	35	34
Total alive	45	45	44	44	44	44	44	44	42
0.3% AA									
Number with lesion	0	0	0	0	1	3	0	1	4
Number at risk	39	39	39	39	39	38	35	35	34
Total alive	39	39	39	39	39	39	39	39	39
5% AA									
Number with lesion	0	0	0	1	1	0	0	1	1
Number at risk	38	38	38	38	37	36	36	36	34
Total alive	38	38	38	38	38	38	38	38	37
10% AA									
Number with lesion	0	0	0	0	0	1	0	1	1
Number at risk	40	40	40	40	40	40	39	39	38
Total alive	40	40	40	40	40	40	40	40	40

Table 3. (*Continued*)

	Readings								
	1	2	3	4	5	6	7	8	9
				Lesions ≥6 mm					
Trend	0.00	0.00	0.00	−2.47	−9.71	−8.18	−19.35	−.71	−7.81
Trend sum	0.00	0.00	0.00	−2.47	−12.19	−20.36	−39.71	−40.43	−48.24
Z*				0.43	1.22	1.51	2.44	2.22	2.32
P (one-tailed)				0.3	0.1	0.07	0.007	0.015	0.01
				Lesions ≥10 mm					
0.0% AA									
Number with lesion	0	0	0	0	1	0	4	1	1
Number at risk	45	45	44	44	44	43	43	39	37
Total alive	45	45	44	44	44	44	44	44	42
0.3% AA									
Number with lesion	0	0	0	0	0	1	3	1	1
Number at risk	39	39	39	39	39	39	38	35	34
Total alive	39	39	39	39	39	39	39	39	39
5% AA									
Number with lesion	0	0	0	0	0	0	0	2	1
Number at risk	38	38	38	38	38	38	38	38	35
Total alive	38	38	38	38	38	38	38	38	37
10% AA									
Number with lesion	0	0	0	0	0	1	0	0	0
Number at risk	40	40	40	40	40	40	39	39	39
Total alive	40	40	40	40	40	40	40	40	40
Trend	0.00	0.00	0.00	0.00	−3.74	2.78	−25.30	−5.34	−6.60
Trend sum	0.00	0.00	0.00	0.00	−3.74	−.96	−26.26	−31.60	−38.20
Z*					0.91	0.14	2.06	2.09	2.28
P (one tailed)					0.2	0.4	0.02	0.02	0.01

† The number of mice with no lesions of the specified size at the preceding reading.

the *i*th group of the series with different amounts of the additive to the diet (vitamin C)—that is, the number without a lesion of the selected size at the beginning of the interval and alive at the end of the interval. $N = \Sigma N_i$ is the total number of the mice. O_i is the number of these mice in the *i*th group that had developed such a lesion during this interval, $O = \Sigma O_i$ is their total for the series, and O/N is the average fraction for the whole series of the mice at risk that had developed the first lesion during this interval.

We next calculate the number of mice in each group that would be expected to develop a first lesion if the additive had no effect. The expected number of mice at risk with new lesions in each group is given by the equation

$$E_i = \frac{N_i O}{N}. \quad [1]$$

The quantity $O_i - E_i$ is then the difference between the observed and the expected number of mice with new lesions in the *i*th group.

These quantities $O_i - E_i$ are then weighted by multiplication by the respective dosages of the preventive substance, D_i:

$$A_i = D_i(O_i - E_i). \quad [2]$$

The quantities, A, B_i, B, C_i, C, and Q are defined as

$$A = \Sigma A_i \quad [3]$$

$$B_i = D_i E_i \quad [4]$$

$$B = \Sigma B_i \quad [5]$$

$$C_i = D_i^2 E_i = D_i B_i \quad [6]$$

$$C = \Sigma C_i \quad [7]$$

$$Q = C - \frac{B^2}{E}. \quad [8]$$

The trend statistic T is then

$$T = A. \quad [9]$$

The variance V of the trend statistic is

$$V = \frac{Q(N - E)}{N - 1} \quad [10]$$

with $E = \Sigma E_i$.

In the calculation of the significance of a number of readings, use is made of other quantities—the trend sum T^* and the variance sum V^*, calculated by

$$T^* = \Sigma T \quad \text{and} \quad [11]$$

$$V^* = \Sigma V. \quad [12]$$

In our tables these sums are taken over the series of readings. The standardized test statistic for trend, Z^*, is

$$Z^* = T^* V^{*-\frac{1}{2}} \quad [13]$$

This quantity varies essentially as a normal distribution, and it represents the number of standard deviations, $V^{*\frac{1}{2}}$, of the observed trend statistic T^*. The value of P (one-tailed) is the area of the standardized normal distribution curve beyond Z^*.

Effect of Vitamin C. Values of the development of first lesions of various sizes in the groups receiving chow alone or chow plus 0.3%, 5%, or 10% ascorbic acid at each of the nine readings

are given in Table 3. Lesions are included only if they were retained. The results of applying the IARC statistical treatment are also given in the table. Every one of the 25 non-zero values of the trend sum is negative, a clear indication that the addition of ascorbic acid to the diet delayed the formation of the lesions. The statistical significance is high, 11 of the values of P being ≤ 0.01. For lesions 4, 6, or 10 mm and larger, four of the P values are ≤ 0.01. Those lesions are mostly malignant, and we conclude that this study provides strong indication that addition of vitamin C to the food decreased the incidence of skin cancer caused in the strain of hairless mice used in this study by irradiation with ultraviolet light.

DISCUSSION

Comparison with the Earlier Study. The earlier study (10) was carried out with mice of the same strain as the present one and with many of the conditions the same. Differences included the kind of commercial mouse chow used, inclusion of nonirradiated controls for each diet, emphasis on persistent neoplastic lesions, methods of identification and classification of individual lesions, a measurement of food consumption, observation through a window in the irradiation cabinet to verify that the mice received full dorsal exposure and did not pile on top of one another, and the supervision of the health of the mice by the staff veterinarian (R.B.), including the performance of necropsies on all mice in the experiment. The eighth reading in the earlier study was made the same length of time after the period of irradiation as the ninth (last) reading in the present study. In Fig. 1 we have plotted for mice in the control groups and groups receiving vitamin C the probability, corrected for unrelated deaths by the Kaplan–Meier method (19), of not having developed lesions ≥4 mm in diameter at this same time following the end of the irradiation. The four points (squares) for the present study agree moderately well with the points (open or solid circles) for the earlier study. Addition of L-ascorbic acid to the chow of these mice greatly decreased the number of mice that had developed skin cancer 14 weeks after the end of the period of irradiation.

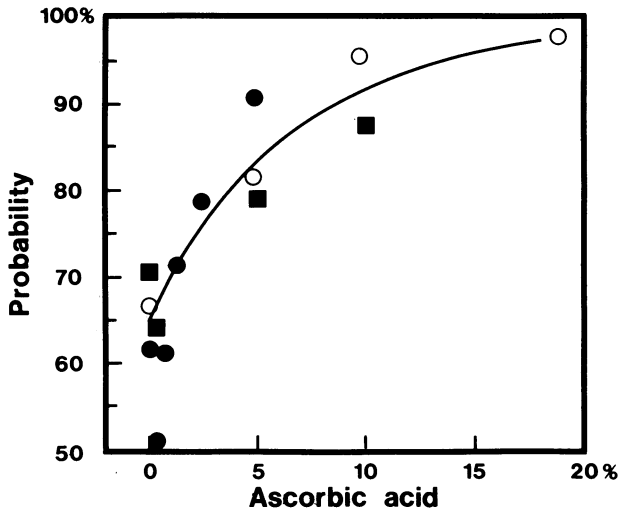

FIG. 1. Probability (with Kaplan–Meier correction for unrelated deaths) of surviving 14 weeks after the end of the period of irradiation without developing any lesions ≥4 mm in diameter as a function of the amount of vitamin C added to the food. ■, Present experiment; ●, Exp. II, ref. 10; ○, Exp. III, ref. 10. The curve serves to guide the eye.

Conclusion. The results reported in this paper show, with high statistical significance, that the addition of L-ascorbic acid to the food (Purina certified rodent chow no. 5002) of hairless mice decreases the incidence of dermal neoplasms (papillomas, squamous cell carcinomas) following a 15-week course of exposures to ultraviolet radiation. The effect found by Black and Chan (12) with a mixture of four antioxidants is probably caused in part by the L-ascorbic acid in the mixture. The present results confirm those reported in the earlier study, in which the basic food was Wayne Lab-Blox (10).

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