Prevention of Experimental Oral Cancer by Extracts of Spirulina-Dunaliella Algae

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Abstract

An extract of Spirulina-Dunaliella algae was shown to prevent tumor development in hamster buccal pouch when a 0.1% solution of 7,12-dimethylbenz[a]anthracene (DMBA) in mineral oil was applied topically three times weekly for 28 weeks. The algae extract was delivered by mouth in continued dosages of 140 μg in 0.4 ml mineral oil three times per week. After 28 weeks, the animals given vehicle and untreated controls all presented gross tumors of the right buccal pouch.

Animals fed canthaxanthin presented a notably and statistically significant reduction in tumor number and size compared with controls. Animals fed β-carotene demonstrated a smaller but statistically significant reduction in tumor number and size. The algae animals presented a complete absence of gross tumors. However, microscopic sections of the buccal pouch in the algae group showed localized areas of dysplasia and early carcinoma-in-situ undergoing destruction.

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Introduction

Hamster buccal pouch carcinogenesis, which was induced by the topical application of a 0.5% solution of 7,12-dimethylbenz[*a*]anthracene (DMBA) in oil, has been shown to be significantly inhibited by the following: a) 13-*cis*-retinoic acid (1), retinyl acetate (2), β-carotene (3), and vitamin E (4,5), b) prostaglandin inhibitors such as aspirin (6), indomethacin (6), and ibuprofen (7), c) immunoenhancers (8) such as bacillus Calmette-Guerin (9) and Levamisole (10), and d) food extracts such as protease inhibitors (11) and onion extract (12). Although tumor development was inhibited in this experimental model, it could not be prevented because 0.5% DMBA is an extremely potent carcinogen and is effective as both an initiator and promotor (13,14). In this tumor system, the DMBA is applied by brush thrice weekly and tumors develop in a consistent sequence of events. Dysplasia develops at 6–8 weeks, carcinoma-in-situ at 8–10 weeks, proliferative and invasive epidermoid carcinomas at 10–12 weeks, and large papillary carcinomas at 12–14 weeks (15).

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Using an experimental hamster buccal pouch model with three applications a week of a 0.1 percent solution of DMBA in oil, the entire time sequence of tumor development was retarded so that gross malignancies developed at 28–30 weeks rather than at 12–14 weeks. In this system, vitamin E was found to completely prevent the development of gross tumors; this was when it was given orally in a dose of 10 mg three times/week on days alternate to the DMBA painting (16).

Because β-carotene and Spirulina-Dunaliella extract were found to inhibit carcinogenesis in the standard hamster buccal pouch model (17), we wondered whether it could completely prevent tumor development in the low dose DMBA model, as did vitamin E. An experimental design was prepared to test this hypothesis. In addition to control groups and algae experimental groups, other experimental groups received β-carotene and canthaxanthin. The algae extract is rich in β-carotene and also contains other carotenoids, vitamin E, phycocyanin, and other constituents (17). β-Carotene is known to convert metabolically to retinoid and is considered a precursor of vitamin A (18). Canthaxanthin is a carotenoid that does not convert and would thus retain all of its carotenoid activity (19).

Materials and Methods

One-hundred noninbred adult male hamsters *(Mesocricetus auratus)* were divided into five equal groups of 20 animals. The hamsters were random bred (Lakeview strain LVG, Charles River Breeding Laboratories, Wilmington, MA) and were fed standard Purina Laboratory pellets and water ad libitum. The pellets (Purina rat chow 5012) contained 4.3 parts per million of carotene. In all five groups, the right buccal pouches were painted three times/week for 28 weeks with a 0.1% solution of DMBA (Sigma Chemical, St. Louis, MO) in heavy mineral oil USP using a no. 3 sable brush.

Animals were divided into the following: two control groups and three experimental groups.

Group 1—Untreated control.
Group 2—Vehicle control, receiving mineral oil.
Group 3—Receiving Spirulina-Dunaliella algae extract (Phycotene) in mineral oil.
Group 4—Receiving β-carotene in mineral oil.
Group 5—Receiving canthaxanthin in mineral oil.

The algae extract (Phycotene Division of Biogenics, Santa Cruz, CA), β-carotene (Sigma Chemical), and canthaxanthin (Hoffman-LaRoche, Nutley, NJ) were administered three times/week by mouth in a dosage of 140 μg (1.5 mg/kg body wt) in 0.4 ml of mineral oil, delivered by pipette, on days alternate to the DMBA applications. The solutions of algae extract, β-carotene, and canthaxanthin were prepared freshly each week and kept in light-protected bottles covered with aluminum foil. When not in use, the bottles were kept in a refrigerator.

The extract of Spirulina-Dunaliella algae was found to contain at least 15 different carotenoids, as seen by two-dimensional chromatography. The major carotenoids were zeaxanthine (20–25%), myxoxanthophyll (10–15%), β-carotene (15–30%), echinenone (10–15%), and β-cryptoxanthin (20–25%). The approximate relative percentages of each carotenoid were evaluated by densitometry, and the total carotenoid content was calculated on the basis of the content and percentage of the carotenes.

The extract used in this experiment was prepared in this laboratory by placing 10 g of the Spirulina-Dunaliella material in a 100-ml solution composed of ether, chloroform, and 100% ethyl alcohol (1:5:4). The solution and material was mixed at 4°C overnight (20 hrs). The solution was then centrifuged at 500 rpm in 50-ml conical tubes to produce a supernatant. The supernatant was then centrifuged for 10 hours at 40,000 rpm at 4°C for the

removal of fine undisclosed material (LB-M ultracentrifuge, Beckman Instruments, Wakefield, MA). The solution was allowed to evaporate under a laminar flow hood for two to three days. The residue was assayed for carotenoid content following solution in 100% ethyl alcohol. Concentration of carotenoid was assessed using a spectrophotometer absorbance and extinction coefficient (E 1% = 2,200–2,600 at 464–497 nm). Approximately 350 μg/ml were calculated for the solution. At least 15 different carotenoids have been observed by two-dimensional chromatography on thin-layer plates with Silica gel G, which was developed with an acetone-hexane mixture (1:19) (vol/vol) as one solvent and an ether-hexane mixture (1:1) as the other. The wavelengths at which carotenoids were measured were 464–497 nm. The standards used were canthaxanthin 464 nm and β-carotene 497 nm with extinction coefficients of 2,200. Other carotenoids' wavelengths ranged from 464 to 490 nm. Spectrophotometry determined the types of carotenoids. The figures for concentrations and percentages are only approximate.

All hamsters were housed five to a cage and maintained in a controlled environment under standardized temperature (24°C) and humidity (35%) conditions with an alternating 12:12-hour light-dark cycle. The animals were weighed initially and then monthly thereafter. The initial age was 60–90 days; weights were 96–120 g. Neither restraints nor anesthesia were used during the application of carcinogen or the delivery of experimental nutrients. From 16 weeks onward, the left buccal pouches of Group 1 and Group 2 animals were examined weekly for evidence of tumors. All animals were killed in a carbon dioxide chamber after 28 weeks, when the tumors in the Group 1 animals were obvious and were moderately large in size.

Pouches were photographed, and the tumors were counted and measured. Data were developed for the expression of mean tumor burden (mean number of tumors × mean tumor volume). The tumor volume was $\frac{1}{3}\pi r^3$ if the tumors were considered to be spherical in overall configuration. The pouches were excised, fixed in 10% formalin, sectioned in paraffin, and stained with hematoxylin and eosin. Autopsies were performed on all animals, and the major organs (heart, lungs, liver, kidneys, adrenals, and spleen) were removed for histological study. They were fixed in 10% formalin, sectioned in paraffin, and stained with hematoxylin and eosin.

Results

Gross Observations

The Group 3 animals (algae extract) were larger and weighed more than the animals in the other groups did (Table 1). All animals in the control groups (Groups 1 and 2) showed gross tumors of the right buccal pouch. The number and size of the tumors varied (Table 2). The animals in Group 3 (algae extract) demonstrated no gross tumors of the right buccal pouches (Table 2). The animals in Group 5 (canthaxanthin) demonstrated very few tumors; in fact,

Table 1. Mean Animal Weights at Termination of Experimental Period[a]

Group		Weights, g
Group 1	(untreated control)	143 ± 20.0
Group 2	(vehicle control)	152 ± 21
Group 3	(algae extract)	192 ± 38
Group 4	(β-carotene)	168 ± 21
Group 5	(canthaxanthin)	154 ± 18

a: Values are means ± SD.

Table 2. Tumor Size, Number, and Burden

Group	No. of Animals at Risk	No. of Animals With Gross Tumors	Mean No. of Tumors per Animal	Mean Diameter of Tumors per Animal, mm	Mean Tumor Burden[a,b], mm^3
1	20	20	3.5	3.6	85.5
2	20	20	3.7	3.9	114.9
3	20	0	0		0
4	20	11	1.8	3.8	51.7
5	20	2	0.5	1.8	1.5

a: Mean tumor burden, mean number of tumors × mean tumor volume ($\frac{4}{3}\pi r^3$ where r is ½ mean tumor diameter).

b: Values for mean tumor burden in Groups 3, 4, and 5 are all significantly different when compared with Control Groups 1 and 2, using Student's t test ($p \leq 0.001$). Figures for mean tumor burden in Groups 3 and 5 are significantly different when compared with Group 4 ($p \leq 0.001$). The difference between Control Groups 1 and 2 is not significant.

most of the animals were free of buccal pouch tumors (Table 2). Most of the animals in Group 4 (β-carotene) demonstrated buccal pouch tumors, but the tumors were fewer in number and smaller in size than those in the controls (Groups 1 and 2). These results could be expressed as mean tumor burden (Table 2). All major organs appeared normal at autopsy.

Microscopic Observations

The right buccal pouches of the animals in Groups 1 and 2 (control groups) presented proliferative epidermoid carcinomas with invasiveness into underlying connective tissue (Figures 1 and 2). In addition, there were numerous foci of hyperkeratosis with dysplasia and

Figure 1. Epidermoid carcinoma in control animal. Hematoxylin and eosin stain. Magnification ×70.4.

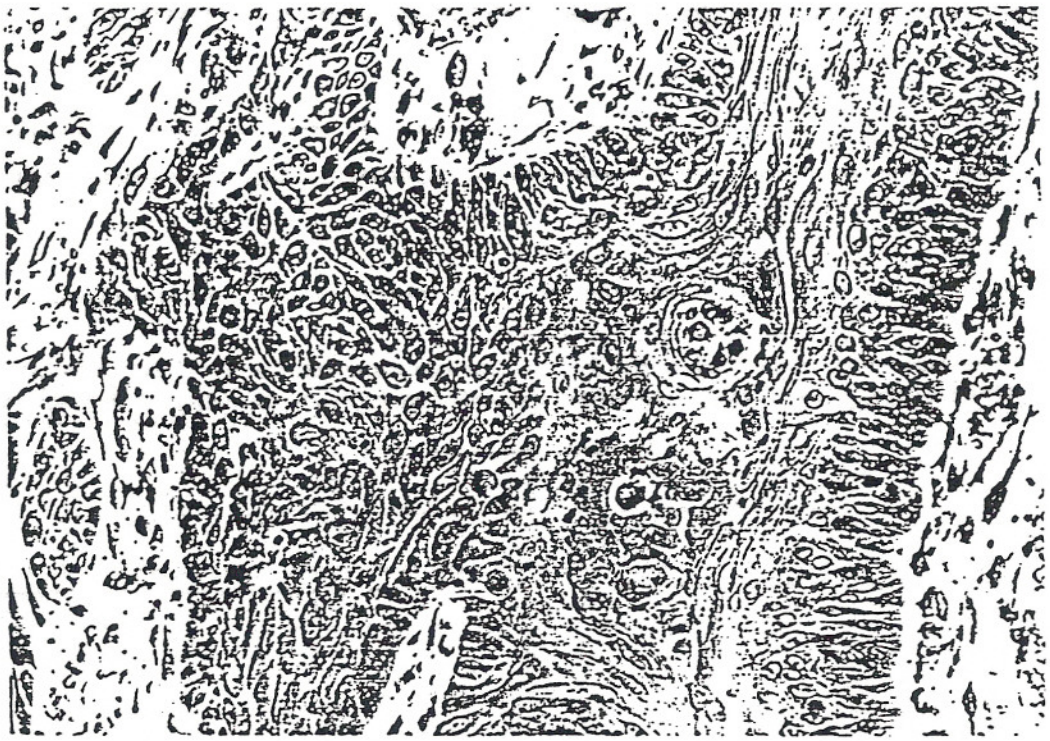

Figure 2. High-power view of Figure 1 showing well-differentiated epidermoid carcinoma, with cellular pleomorphism and numerous mitoses. Hematoxylin and eosin stain. Magnification ×308.

carcinoma-in-situ. Some lymphocytic infiltration was noted in the areas of tumor invasion and carcinomas-in-situ.

Group 3 animals (algae) presented no frank carcinomas; however, scattered foci of dysplasia and carcinoma-in-situ undergoing degeneration and cellular destruction were seen (Figures 3 and 4). There were dense accumulations of lymphocytes and monocytes (histiocytes) in the underlying connective tissue, often close to the areas of dysplasia. In some pouches, the lymphocytic accumulation was so dense in localized areas that it resembled lymphoid tissue with lymph nodule configurations. Where the lymphocytic infiltrate was light, it was often seen in a perivascular distribution.

Group 4 animals resembled those in Groups 1 and 2, except that the carcinomas appeared smaller and somewhat less invasive. Lymphocytic infiltration was more pronounced than in Groups 1 and 2. Group 5 animals resembled Group 3 animals histologically, with dense lymphocytic infiltration, scattered foci of dysplasia, and carcinoma-in-situ undergoing degeneration. The major organs were normal at autopsy in all groups.

Discussion

The algae extract, which was administered by mouth, prevented tumor formation in this hamster buccal pouch experimental model. Although carcinomas were beginning to develop as microscopic foci, they were being destroyed, probably by an immune response. The evidence for this concept is the dense lymphocytic-monocytic infiltrate. This response is similar to that seen in gross epidermoid carcinomas undergoing regression after local injection into the buccal pouches of algae extract, β-carotene, canthaxanthin, or α-tocopherol (20). The monocytes were found to be cytotoxic to tumor target cells *in vitro*

Figure 3. Carcinoma developing in an area of leukoplakia in algae animal. Carcinoma presents evidence of destruction, and underlying corium is infiltrated with lymphocytes. Hematoxylin and eosin stain. Magnification ×176.

when obtained from the peritoneum, and the macrophages (locally) were positive for tumor necrosis factor alpha. The lymphocytes (locally) were T-cells (determined by immunohistochemistry). These observations support the concept that the algae extract can prevent cancer development by stimulating an immune response to selectively destroy small initial foci of developing malignant cells.

The effectiveness of the canthaxanthin in cancer prevention and the reduced effectiveness of the β-carotene can possibly be explained by the fact that the β-carotene may have been partially converted to retinoid in the gastrointestinal tract of the animals, whereas the canthaxanthin was not. Thus, the canthaxanthin offered the more potent carotenoid effect, as demonstrated in previous studies, whereas the β-carotene offered a less-effective retinoid effect in addition to a carotenoid effect. In rats, there is the enzyme β-carotene 15,15-dioxygenase that converts β-carotene into vitamin A. This enzyme has been found in the intestine, liver, and other tissues (21,22). The enzyme may be absent in hamster buccal pouch. In previous studies using local injection of β-carotene and canthaxanthin into the tumor site, the β-carotene was more effective in regressing tumors than was the canthaxanthin (23). It seems reasonable to assume that the β-carotene in the hamster buccal pouch tissue did not convert to retinoid, which would reduce its effectiveness as an antitumor agent. In addition, histological analysis disclosed that canthaxanthin produced a more profound lymphocytic response in the hamster pouch compared with β-carotene. This effect agrees favorably with recent *in vitro* evidence showing that canthaxanthin *in vitro* was a superior immunoenhancer, independent of vitamin A effect and of T- and B-cell responsiveness (compared with β-carotene) (24).

The tumor-inhibitory effect of dietary β-carotene has been demonstrated by Rettura and

Figure 4. High-power view of cancer site undergoing cellular destruction *(arrows)*. Hematoxylin and eosin stain. Magnification ×308.

colleagues (25) in another animal model. Stich and others (26,27) found that dietary vitamin A and β-carotene exerted a protective effect against the carcinogenic effect of betel nut/tobacco chewing. Canthaxanthin was found to lack this protective activity, suggesting a mechanism of protection other than scavenging of free radicals. The protection effects of vitamin A and β-carotene were evaluated by fewer exfoliated cells with micronuclei in exfoliated oral mucosal cells.

All animals in this study were fed the same diet ad libitum. The gain in body weight was not statistically significant and was probably due to a better overall health status. A direct quantitation of the amount of food eaten by these animals was not ascertained, but with daily observation of the animals, it did not appear that more food was eaten.

There was no evidence of toxicity in any of the animals receiving the algae extract. Gross and microscopic study of major organs revealed no evidence of chronic toxicity after 28 weeks. Previous studies in this laboratory also showed no evidence of toxicity after 30 weeks.

The effectiveness of the algae extract by oral administration in hamsters indicates that further research is warranted in other species and other experimental tumor models. The chemoprevention of cancer may become a significant approach to the future management of cancer (28), and micronutrients could possibly play a major role in cancer prevention.

Acknowledgments and Notes

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publ. No. NIH 85-23, revised 1985). This study received support from Biogenics (Santa Cruz, CA).

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