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Antineoplastic Properties of Maharishi-4 Against DMBA-Induced Mammary Tumors in Rats[1]

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SHARMA, H. M., C. DWIVEDI, B. C. SATTER, K. P. GUDEHITHLU, H. ABOU-ISSA, W. MALARKEY AND G. A. TEJWANI. *Antineoplastic properties of Maharishi-4 against DMBA-induced mammary tumors in rats.* PHARMACOL BIOCHEM BEHAV **35**(4) 767–773, 1990.—Maharishi-4 (M-4), an ayurvedic food supplement, was tested for anticarcinogenic and anticancer properties against 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats. The 6% M-4-supplemented diet protected DMBA-induced carcinogenesis by reducing both tumor incidence and multiplicity during initiation and promotion phases. The control animals who developed tumors when supplemented with M-4 diet for four weeks showed tumor regression in 60% of cases. There was no significant difference in the food intake or weight gain in rats who were on M-4-supplemented diet compared to control group. Possible mechanisms of action of M-4 are discussed.

Antineoplastic Maharishi-4 DMBA Mammary tumors Opioid peptides Prolactin Estrogen Progesterone receptors

AYURVEDA (ayu = life, veda = knowledge, meaning science of life) is an age old science of life which is being revived in its complete form under the name of Maharishi Ayurveda. Maharishi Ayurveda deals with the four areas of life: 1) Mind and consciousness, 2) physiology, 3) behavior, and 4) environment (6). Rasayanas are groups of herbal preparations which bring about homeostasis or balance in physiology, enhance immunity, and retard aging (15).

In ayurvedic literature M-4 is described to be an effective rasayana for the treatment of carcinoma, gastrointestinal disorders, atherosclerosis and to bring tranquility of the mind (15). However, to date there has been no scientific investigation of the properties of M-4. The purpose of this investigation was to test the anticarcinogenic and anticancer properties of M-4 against 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats. In view of the role of the prolactin, estrogen, opioid peptides, and estrogen and progesterone receptor in the tumor, these parameters were measured to determine the possible mechanism of action of M-4 (3, 4, 14).

METHOD

M-4 was supplied by Maharishi Ayurveda Products International, Stoneham, MA 02180. The ingredients in M-4 are: Indian gallnut, Indian gooseberry, dried catkins, Indian pennywort, nutgrass, white sandalwood, evalyulus alsinoides, embella, aloewood, licorice, cardamom, cinnamon, cyperus, turmeric, honey, raw sugar and ghee (clarified butter). The exact composition of various ingredients in M-4 was not disclosed by the supplier, but the quality control (e.g., minimal variation from batch to batch) was assured. For the supplementation of rat diet with this substance the M-4 was mixed with rodent powdered chow (Wayne Pet Food Division, Chicago, IL) in a ratio of 6% (w/w). This ratio was extrapolated from the human dose for M-4. The analysis of the different components of M-4 has revealed large number of constituents including antioxidants, e.g., tannic acid, flavinoids, catechin, alpha tocopherol, polyphenols, ascorbate, riboflavin, and beta carotene (16–29).

Carcinogenic protocol was used as described by Abou-Issa *et al.* (1). Fifty-day-old female rats of Sprague-Dawley strain (pathogen free, SASCO, Inc., Omaha, NE) were randomly assigned to five groups (20 rats per group). The experimental groups are described in Table 1. All animals were allowed to have food and water ad lib. All animals were given DMBA (Sigma Chemical

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TABLE 1

Experimental Groups*	
Control group (C)	Normal diet throughout the experiment.
Initiation group (I)	M-4-supplemented diet 1 week before and 1 week after DMBA treatment.
Promotion group (P)	M-4-supplemented diet one week after DMBA treatment until the end of the experiment.
Promotion & Initiation group (I+P)	M-4-supplemented diet from 1 week before DMBA treatment until the end of the experiment.
Regression group (R)	Control animals after 18 weeks who had developed tumors were put on M-4-supplemented diet for 4 weeks.

*All the animals received one dose of DMBA 75 mg/kg body weight.

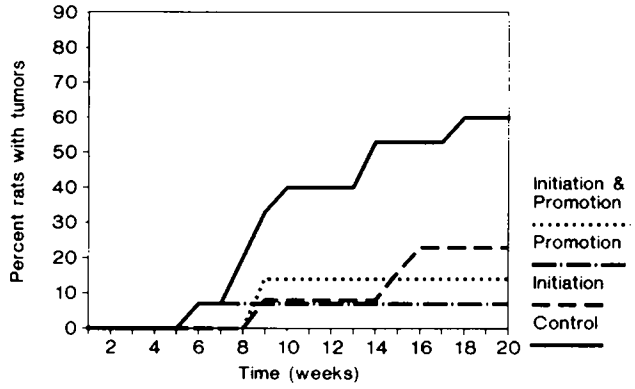

FIG. 1. Effect of 6% M-4-supplemented diet on tumor incidence. Tumor incidences in all groups were statistically analyzed using Chi-square test. C = 60%, I = 21%, P = 7%, I&P = 14%. I, P and I+P group were significantly different from C group ($p<0.05$).

Company, St. Louis, MO,approximately 95% pure; 75 mg/kg in 1 ml of sesame oil) by gavage after being on the diet for one week. Rats were weighed and examined weekly for the presence of mammary tumors for a period of 18 weeks after DMBA administration. For the regression group, ten rats having varying size of tumor from the control group were placed on a 6% M-4-supplemented diet. Tumor size was measured in all 10 rats before placing them on 6% M-4-supplemented diet and then measured once a week for four weeks. The tumors were measured once a week with micrometer caliper (12). Two measurements were taken, the largest dimension and the dimension at the right angle of largest dimension. Tumor volume was calculated by the formula: Tumor volume = $[(\text{width})^2 \times \text{length}]$.

At the termination of the experiment, all rats were sacrificed by decapitation. Blood was collected in tubes containing 0.1 vol. of 80 mM EDTA and 0.5% bacitracin (9:1) for the determination of plasma prolactin, estradiol and β-endorphin. Pituitary and hypothalamus were collected in liquid nitrogen, and stored at −70°C. Beta-endorphin and Met-enkephalin levels were estimated by a sensitive and specific RIA procedure developed as described earlier (30, 31, 33). Samples for histological examination were taken from the mammary tumor, liver, spleen, stomach, pancreas, kidney, heart and lung. A part of tumor was stored in liquid nitrogen for estrogen and progesterone receptor assays. The estrogen and progesterone receptors in the tumor tissue were done by the multipoint dextran-coated charcoal method (14). Prolactin and estradiol assay were done by radioimmunoassay (9,13).

Histopathology

All the tissues were fixed in 10% buffered formalin, processed through Fisher histomatic processor, and embedded in paraffin. Three micron sections were cut and stained with hematoxylin and eosin for microscopic examination.

Statistical Analysis

Data were statistically analyzed using Chi-square and Student's *t*-test utilizing EPISTAT computer program.

RESULTS

The effect of 6% M-4-supplemented diet on the incidence of DMBA-induced tumors is given in Fig. 1. There were some deaths in each group. Tumor incidence was calculated from the surviving rats and the number was rounded off to the nearest full number. The data are as follows:

Group	Number of Animals With Tumors	Total Number of Surviving Animals
Control (C)	9/15	(60%)
Initiation (I)	3/13	(23%)
Promotion (P)	1/14	(7%)
Initiation and Promotion (I&P)	2/14	(14%)

During promotion (P) phase tumor incidence was reduced to 7% in comparison to 60% incidence in control group which is an 88% drop in the incidence of tumor in this group. Similarly, a 77% decrease in tumor incidence was observed in I&P group (a drop from 60% of control group to 14% in I&P group). But only 60% reduction in tumor incidence was observed in I phase (a drop from 60% of control group to 23%).

M-4-supplemented diet also showed reduction in the number of DMBA-induced tumors per animal (tumor multiplicity). The data on tumor multiplicity are shown in Fig. 2. A 69, 85 and 85% reduction in number of tumors per animal was observed in I, P and I+P groups respectively. The number of tumors per rat in I, P and I+P group was significantly different ($p<0.05$) from control group. The effect of M-4-supplemented diet on tumor volume in the regression group is given in Table 2. M-4-supplemented diet provided tumor regression in 60% of rats. In 50% of these rats the tumors regressed completely. If the tumor had reached a critical size it kept growing. A control for the regression group in this study was regrettably not included. However, in another similar experiment in the control group, no regression in tumor size was observed during the period of 4 weeks.

The animals in various experimental groups were weighed every week. In order to simplify the presentation of data, only last week's weight gain is reported. The weight gained by the rats on M-4-supplemented diet is as follows: C = 31.2%, I = 32.7%, P = 34.0%, I&P = 31.9%. There was no significant difference in weight gain of rats among all the groups throughout the duration of

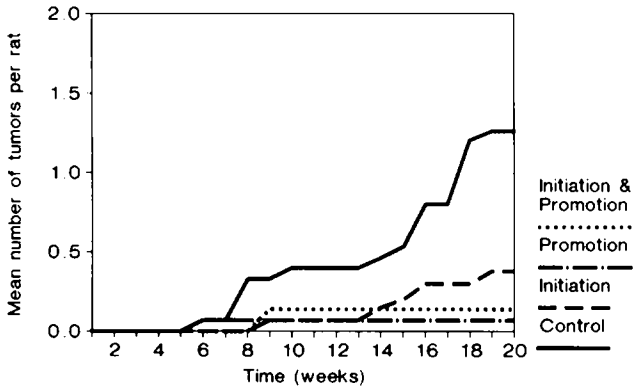

FIG. 2. Effect of 6% M-4-supplemented diet on tumor multiplicity. Tumor multiplicity in all groups was statistically analyzed using two-tailed Student's *t*-test. The average number of tumors/animals is: C = 1.3, I = 0.4, $p = 0.1$, I&P = 0.1. I, P and I+P group was significantly different from C group ($p<0.05$).

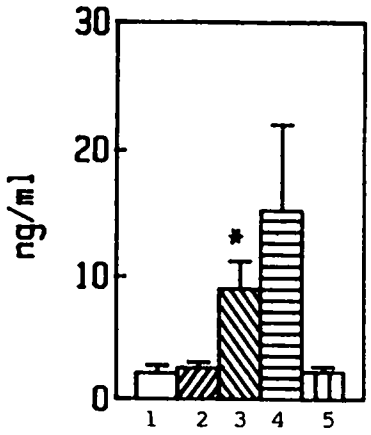

FIG. 3. Effect of M-4 on the levels of plasma prolactin in different experimental groups (1: control, 2: initiation, 3: promotion, 4: initiation and promotion, 5: regression). Values are mean ± SEM and number of observations are 3–5. Value is significantly different from control at $p<0.05$ level.

experiment indicating that feeding of M-4 in diet does not produce caloric imbalance.

The plasma prolactin level was also measured in all experimental groups (Fig. 3). No significant differences were observed in the different groups except in the promotion group where the values increased significantly ($p<0.05$). M-4 altered the beta-endorphin and Met-enkephalin immunoreactivity in the pituitary and hypothalamus of the DMBA-induced rats (Fig. 4). Beta-endorphin levels in the pituitary did not change significantly. The beta-endorphin levels decreased in plasma. However, the decrease was not significant except in the promotion group ($p<0.05$). On the other hand, beta-endorphin levels in the hypothalamus significantly decreased with M-4 intake except in the initiation and promotion group where the values were not significant. This may be due to a large variation in the values of this group, even though the decrease was as much as 58% of the control.

The M-4 did not change the levels of Met-enkephalin in the pituitary of DMBA-ingesting animals. However, in the hypothalamus, Met-enkephalin levels increased significantly from that of the control group except in the regression group of animals.

The plasma estradiol level was measured in the control group and initiation and promotion (I+P) group. Control and I+P groups had values of 225.6 ± 22.2 and 177.6 ± 37.7 ng/ml (mean ± SEM, n = 5). There was a 20% nonsignificant decrease in the estradiol level in I+P group. The plasma estradiol levels were not measured in other three groups because of lack of sufficient material. Estrogen and progesterone receptor bindings were assayed in the tumors from rats from control, initiation and the regression groups (Fig. 5). All the tumors were positive for estrogen and progesterone receptors.

Histopathological studies were performed in four mammary tumors from control group. All the tumors were adenocarcinoma (Fig. 6) with mild to moderate areas of inflammation. One tumor showed areas of necrosis and two tumors had areas of fibrosis. In the I group three tumors were examined. All the tumors showed areas of fibrosis and regression except one tumor which had a focus of benign fibroadenoma. In the P group only one tumor was available which was adenocarcinoma with mild to moderate inflammation. In I+P group also only one tumor was available which exhibited extensive fibrosis, regression and benign areas (Fig. 7). Three tumors were examined from the regression group in which rats with tumor from control group were placed on M-4-supplement diet. Two of the adenocarcinomas showed regression with benign centers (Fig. 8). The histology of the heart, lung, kidney, stomach, pancreas was unremarkable in all the groups. Spleen showed follicular hyperplasia in all the groups.

TABLE 2

CHANGES IN TUMOR VOLUME OF THE RATS IN THE REGRESSION GROUP TREATED WITH 6% M-4-SUPPLEMENTED DIET FOR FOUR WEEKS

Rat Number	Tumor Size (CM^3) 1st Week	2nd Week	3rd Week	4th Week	Change in Tumor Volume (CM^3)
1	0.36	0.21	0.03	0	−0.36
2	1.37	1.09	0.67	0.08	−1.29
3	3.16	4.63	6.78	6.78	+3.62
4	6.08	6.08	14.89	16.38	+10.30
5	0.30	0.11	0	0	−0.30
6	14.89	19.65	19.65	27.43	+12.54
7	5.32	6.78	9.84	10.97	+5.65
8	0.36	0.11	0.03	0	−0.36
9	0.36	0.01	0.01	0.01	−0.35
10	0.36	0.21	0.01	0.00	−0.36

\+ Increase in tumor size.
− Decrease in tumor size.

DISCUSSION

The results from this study indicate that in rats 6% M-4-supplemented diet reduced DMBA-induced mammary tumor incidence and multiplicity significantly during initiation phase as well as remarkably during promotion phase. M-4 was also shown to be effective in the regression of fully formed tumors of the control group.

The protocol of Higgins (34) had previously reported higher incidence of DMBA-induced mammary tumors as compared to our studies. However, they have used relatively higher dosage of DMBA (130 mg/kg body weight) which in our experience has

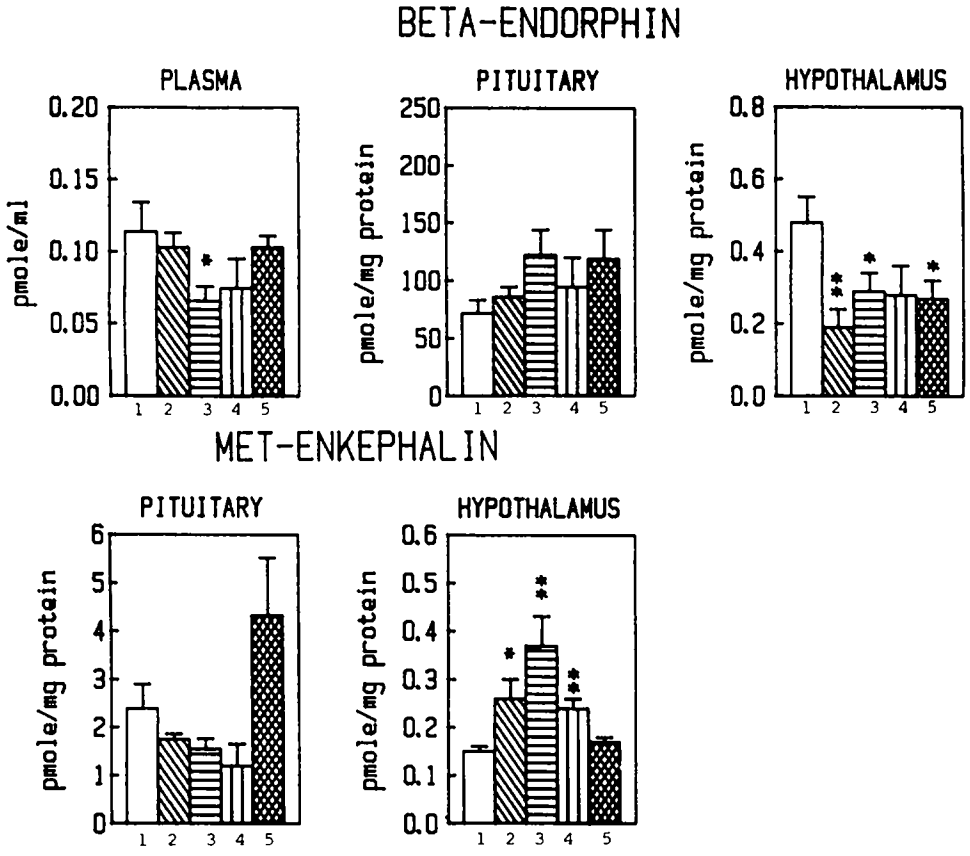

FIG. 4. Effect of M-4 on the levels of beta-endorphin and Met-enkephalin levels in plasma, pituitary and hypothalamus in different experimental groups. 1) Control; 2) initiation; 3) promotion; 4) initiation and promotion; 5) regression group. Values are mean ± SEM and number of observations are 3–6. Values are significantly different from the control group at $*p<0.05$ and $**p<0.01$ levels.

resulted in severe toxic effects (more than half of the animals died within a week). Therefore, in our previous experiments (1), we used a dosage of 75 mg/kg body weight which yielded about 70% mammary tumor incidence in 50-day-old Sprague-Dawley rats. However, in this experiment we got an incidence of 60% which is very similar to our previous observation. Some variation in tumor incidence may be due to different rodent diets and other environmental conditions.

Chemical carcinogenesis appears to exhibit two main phases in the multistep process, an irreversible initiation phase followed by promotion phase (2). Steroid sex hormones and pituitary prolactin are the primary endogenous promoters of mammary carcinogenesis in rats treated with carcinogens (5). Ovarian function and the level of ovarian steroid hormones at the time of polynuclear hydrocarbon carcinogen administration is critical for the development of mammary tumors (4). Aberrations in endogenous sex steroid hormone levels may influence the promotion phase of human breast cancer. Familial or international variations in risk of mammary cancer may be accounted for in part by such variations (32). Ovariectomy alters DMBA-induced mammary tumor growth (35). Also, estrogen and progesterone receptors in the mammary tumor are shown to be important in controlling the tumor growth (12). In this study attempts were made to correlate the tumor incidence with the estrogen levels and receptor function. However, due to lack of adequate samples, complete data on hormone levels and receptor studies are lacking. With the available data it seems that M-4 administration does not significantly influence the circulating estradiol level. Also, all the mammary tumors tested were estrogen and progesterone receptor positive, especially in tumors where M-4-induced regression was observed, suggesting that M-4 may be acting by blocking the cell receptors needed for cellular growth.

Small mammary tumors induced by DMBA in rats show

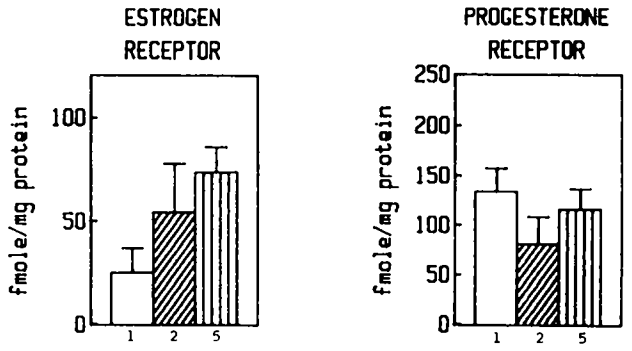

FIG. 5. Estrogen and progesterone receptors in breast tumors in 1) control, 2) initiation, and 5) regression groups. Values are mean ± SEM for at least 2 observations.

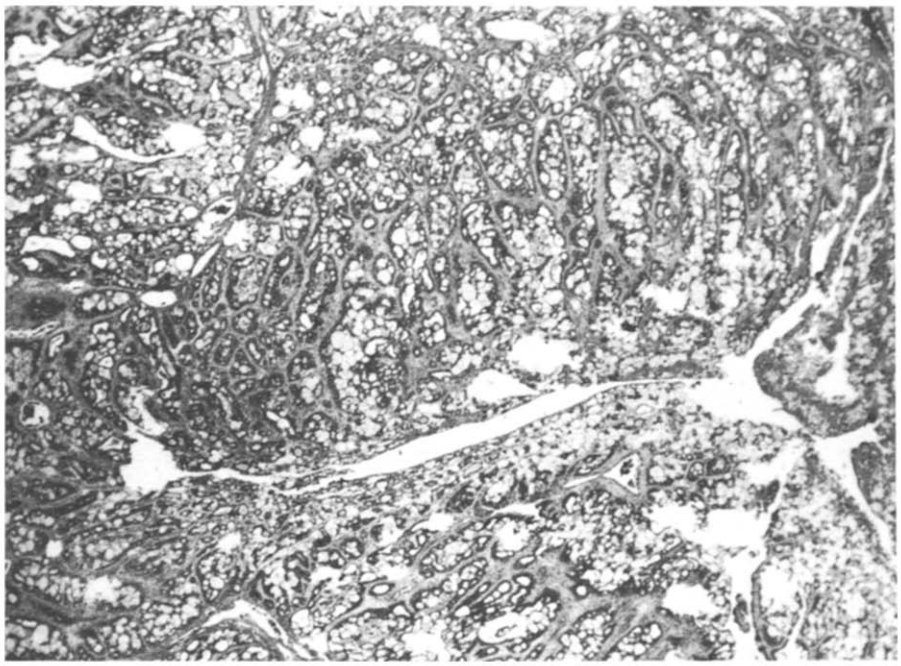

FIG. 6. Mammary adenocarcinoma from control animals showing proliferation of atypical gland in the fibrous stroma. H&E ×158.

homogeneity of histology. However, the larger tumors show histologic variations. The tumors are usually well differentiated adenocarcinomas with fully formed tubular structures. The larger tumors, however, have been described to show areas of papillary formation, cribriform pattern and lipid secretions (35). In our study, tumors from control group could clearly be classified as adenocarcinomas with areas of inflammation. No other features were seen. Tumors from all other groups (I, P, I + P and regression

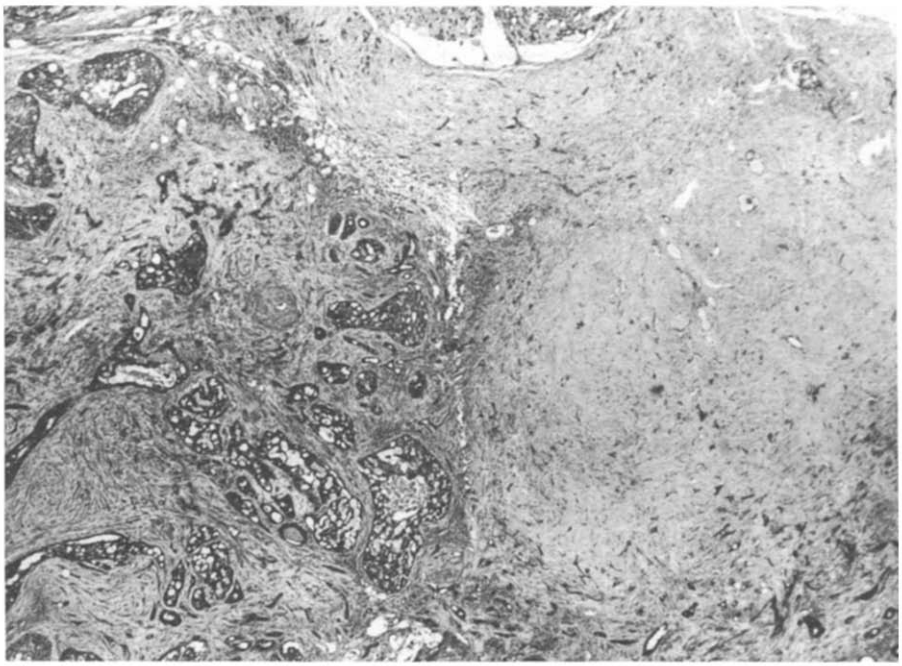

FIG. 7. Mammary adenocarcinoma from I+P group showing areas of maturation and extensive fibrosis representing regression of tumor. H&E ×158.

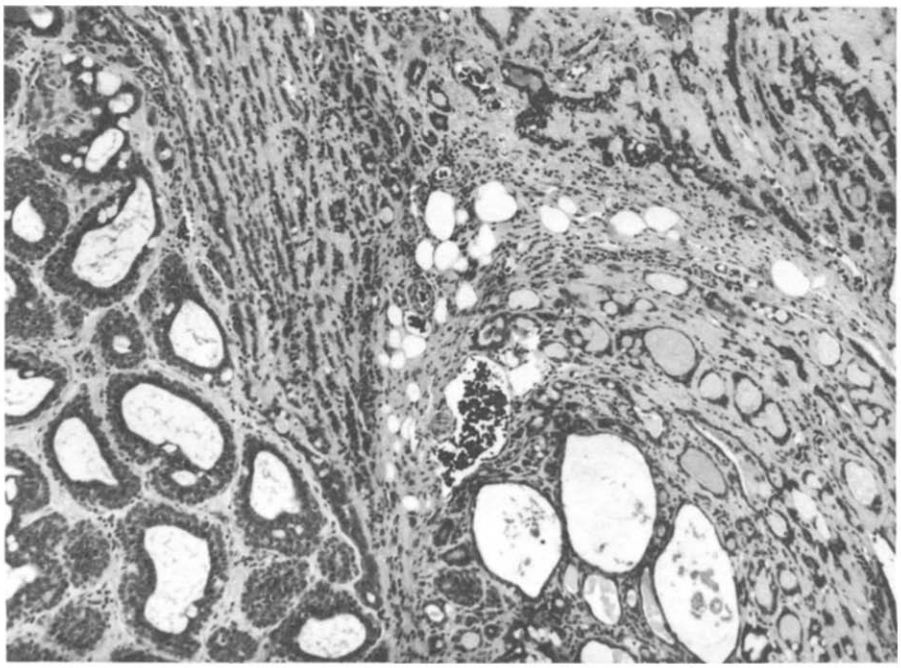

FIG. 8. Adenocarcinoma from regression group showing areas of maturing benign glands with fibrotic background representing differentiation and regression of tumor. H&E ×400.

group) were highlighted by the presence of benign and regression areas suggesting the effect of M-4 on the tumor growth. Histopathological studies indicated that M-4 causes the maturation of the tumor with differentiation towards benign morphology and associated fibrosis causing regression of tumors.

Intracerebroventricular injection of beta-endorphin into rats has been shown to stimulate prolactin secretion within 10 min of injection (8). Rats subjected to stress show an immediate rise in plasma beta-endorphin followed by an increase in the plasma prolactin level (7). High levels of prolactin have been suggested to facilitate mammary carcinogenesis (10) and antiprolactin drugs have been shown to eliminate the enhancement of mammary tumorigenesis (3). Thus, it is possible that M-4 may decrease the incidence of mammary cancer by decreasing CNS beta-endorphin and plasma prolactin levels. We did find a decrease in the hypothalamic beta-endorphin levels in the M-4-treated animals (Fig. 4), however, it was not followed by a decrease in the plasma prolactin levels in the M-4-ingesting rats (Fig. 3). However, we can't rule out the possibility that plasma prolactin may have decreased in rats during the progression of tumors, but at the time of sacrifice of animals (at the end of the experiment) it returned to normal levels (Fig. 3).

It is also possible that other peptides such as Met-enkephalin may be involved in the antineoplastic action of M-4. Met-enkephalin has been shown to inhibit local subcutaneous tumor growth induced by melanoma cells in C57BL/6T mice (11). We have earlier observed that DMBA-induced mammary tumor formation is associated with a decrease of 35% in the striated Met-enkephalin level (36). Thus, it is likely that a decrease in the CNS Met-enkephalin may facilitate carcinogenic process, while an increase in the level of this peptide may inhibit cancer. In support of this hypothesis, we observed that M-4-treated rats showed an increase in the hypothalamic Met-enkephalin levels (Fig. 4). Thus, it appears that antineoplastic action of M-4 may involve changes in the level of opioid peptides in the central nervous system which have been shown to influence carcinogenic events.

Reactive oxygen species produced by activated phagocytes have shown in in vitro culture systems to induce cytogenetic damage in cultured mammalian cells and hamster cells (37). Reactive oxygen species have also been shown to induce both benign and malignant neoplasms in athymic nude mice (38). These data suggest that reactive oxygen species by altering DNA structure of target cells may cause changes in their growth characteristics and induce tumorigenesis (39). Since M-4 analysis shows that it contains multiple antioxidants, it is possible that the antineoplastic effects of M-4 against DMBA-induced mammary cancer may be due to antioxidant effect. This area needs further exploration.

Inhibition of DMBA-induced mammary tumors in rats by M-4, an ayurvedic food supplement, is a provocative finding. Our data have demonstrated that M-4 prevents the chemical carcinogenesis and also is effective in regression of tumors.

ACKNOWLEDGEMENTS

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