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Chemoprevention of 7,12-Dimethylbenz[*a*]anthracene-induced Mammary Carcinogenesis in Rat by the Combined Actions of Selenium, Magnesium, Ascorbic Acid and Retinyl Acetate

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The chemopreventive actions of sodium selenite (SS), magnesium chloride (MC), ascorbic acid (AA) and retinyl acetate (RA), given singly or in combinations, on mammary carcinogenesis induced by 30 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA) in female adult rats were evaluated. Administration of modulators was carried out from the age of 40±3 days to 240±3 days. When DMBA alone was given 100% of the rats developed mammary tumors. When modulators were given singly the tumor incidences were reduced to 51.77% (SS), 46.4% (MC), 57.1% (AA) and 48.1% (RA). When the modulators were given in combination of twos, the tumor incidences were further reduced to 29.5% (SS+MC), 31% (SS+AA), 29.6% (SS+RA), 25.9% (MC+AA), 31.8% (MC+RA) and 34.6% (AA+RA). Administration of modulators in combinations of threes resulted in still further reduction of tumor incidences to 22.2% (SS+MC+AA), 19.2% (SS+MC+RA), 16% (MC+AA+RA) and 23.1% (AA+RA+SS). When all four modulators were given concurrently the tumor incidence was only 12%. Further, the number of tumors per tumor-bearing animal declined with the increase in the number of agents used in combination for modulation.

Key words: Chemoprevention — Mammary carcinogenesis — Retinyl acetate — Ascorbic acid — Selenium and magnesium

It is now known that several naturally occurring as well as synthetic chemical substances are capable of inhibiting chemical carcinogenesis.[1-4] The chemoprevention of carcinogenesis has been accomplished by selenium,[5-9] magnesium,[10-12] ascorbic acid[13,14] and retinoids.[15,16] Since these modulators may inhibit chemical carcinogenesis by different mechanisms it seemed worthwhile to use two or more modulators concurrently or sequentially in the expectation of enhancing the chemopreventive action and also, at the same time, to reduce the possible toxic effects of individual compounds on the host. Already several workers have achieved chemoprevention of carcinogenesis by the combined action of different modulators.[17-19]

The present communication reports the chemopreventive action of different combinations of selenium salt, magnesium salt, ascorbic acid and retinyl acetate on 7,12-dimethylbenz[*a*]anthracene(DMBA)-induced mammary carcinogenesis in rats.

MATERIALS AND METHODS

Animals Randomly bred young (40±3 days old) virgin rats of Sprague-Dawley strain were used for this study. Animals were maintained under controlled environmental conditions of 12 h light and 12 h dark, 50–60% humidity and 20–22°C. They were put on a standard diet (formula: C. R. I., Bombay) whose selenium and magnesium contents were respectively 0.2 ppm and 450 ppm and tap-water (in which, unless otherwise stated, the selenium and magnesium contents were respectively 0.2 ppm and 6.6 ppm) *ad libitum*.

Chemicals DMBA, sodium selenite (Na_2SeO_3), magnesium chloride ($MgCl_2$), L-ascorbic acid and retinyl acetate were purchased from Sigma Chemical Co., USA.

Treatment schedule Fig. 1 depicts the treatment schedule of the animals with the carcinogen and different modulators. Table III gives particulars of the different control and experimental groups. Sodium selenite was added to the daily drinking water at 3 μg/ml, 2 μg/ml and 1 μg/ml sequentially for three specific time periods commencing at the age of 40±3 days. Likewise, magnesium chloride was added to the daily drinking water at 50 μg/ml, 30 μg/ml and 20 μg/ml sequentially for three specific time periods, commencing at the age of 40±3 days. Ascorbic acid was given to the rats of specific groups daily from the age of 40±3 days in steps at 25 mg/ml, 15 mg/ml and 10 mg/ml sequentially in their drinking water. In order to make the sour fluid palatable, sugar (2 g%) was added.[14] Fresh solutions were pre-

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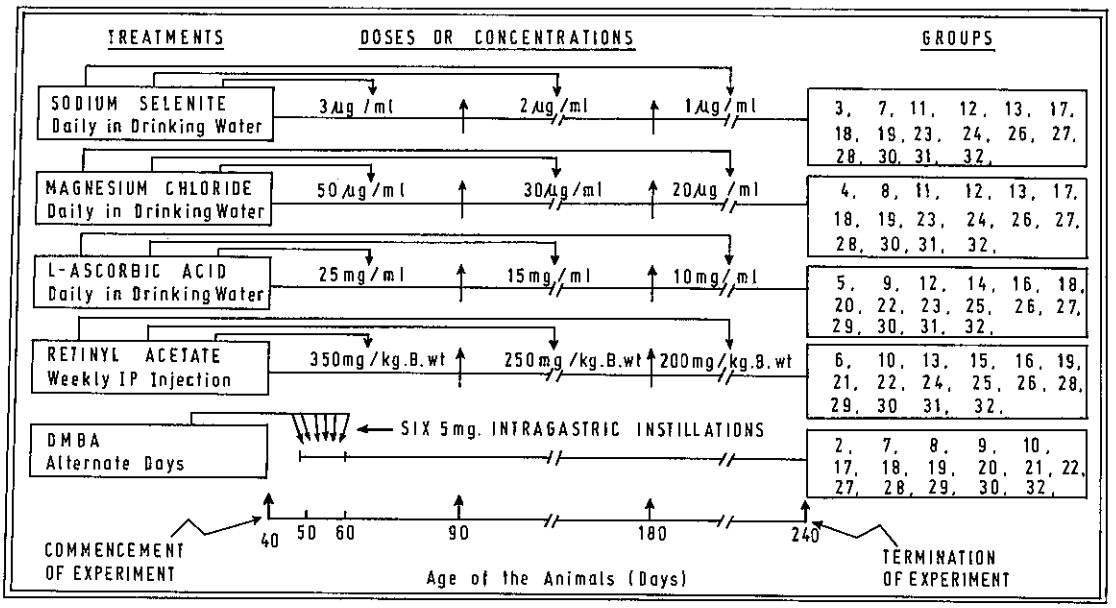

Fig. 1. Treatment schedule of carcinogen and modulators.

Table I. Average Estimated*/Calculated** Intakes of Various Modulators by Rats during Three Specific Periods

Modulator		Age in weeks		
		6–12	13–25	26–34
Sodium selenite*	Conc: (μg/ml)	3	2	1
	Intake: (μg/100 g body wt.)	44.44	27.14	13.36
Magnesium chloride*	Conc: (μg/ml)	50	30	20
	Intake: (μg/100 g body wt.)	740.33	410.32	265.74
Ascorbic acid*	Conc: (mg/ml)	30	20	10
	Intake: (mg/100 g body wt.)	370.5	207.34	134.35
Retinyl acetate**	ip route (mg/kg body wt.)	350	250	200

pared daily in tapwater which was boiled and cooled prior to use. Wherever more than one modulator was to be given by the oral route, the chemicals were added to the same drinking water. The total amount of water consumed during a period of 24 h by all the animals of a given DMBA+modulator(s) group was recorded at weekly intervals by noting down the volume of water provided and the volume of water left undrunk by the rats at the end of the 24 h period. Average volume of water consumed per day was then estimated. As the body weight of each rat in each group was recorded (Table II) at the same weekly intervals, the average body weight of rats in a given group was calculated for all these intervals, and then the average intake of each water-borne modulator per 100 g body weight was estimated. Table I depicts the average "estimated effective doses" (μg or mg/100 g body weight) of the water-borne modulators consumed in 24 h by each rat at three different phases during the observation period. Retinyl acetate was administered once a week by the ip route sequentially at the dose levels of 350 mg/kg, 250 mg/kg and 200 mg/kg for three specific periods to the rats of particular groups starting at the age of 40±3 days. Modulators were given till the animals reached the age of 240±3 days.

DMBA was dissolved in sesame oil (5 mg/0.2 ml oil), and 30 mg of the carcinogen was delivered to each rat of

particular groups by the intragastric route under mild ether anesthesia in 6 equal instalments (i.e. 5 mg per delivery) on alternate days, commencing when the rats attained the age of 50±3 days. Animals were carefully palpated once a week for the detection and documentation of mammary tumors. As and when tumors showed early signs of necrosis they were surgically removed under mild ether anesthesia. All surviving animals were killed at the end of the modulator treatment period and the mammary tumors recovered were processed for histopathological classification into adenocarcinomas and fibroadenomas.[20-22]

The chi-square test was applied for determining the level of significance of differences in tumor incidence between control and experimental groups.

RESULTS

Modulators, given singly or in combinations, did not appear to adversely affect the body weight increase in rats during the observation period (Table II). Body weight changes in DMBA- and DMBA+modulator(s)-treated animals are modified by the occurrence of tumors and the removal of tumors showing early signs of necrosis. Table III lists the number of rats with mammary tumors, mean number of tumors per tumor-bearing animal and also the histopathological types of tumor in different control and experimental groups. Table IV shows the frequencies of tumor incidence at fortnightly intervals in DMBA-treated groups. Fig. 2 shows the

Table II. Body Weight Changes in Control and Experimental Animals

Group	Treatment(s)	Body weight (g) at different ages (weeks): mean±SD							
		6	10	14	18	22	26	30	34
Gr_1	Control	108±11	146±13	168±14	192±16	223±16	246±16	268±17	288±22
Gr_2	DMBA	112±10	139±10	162±17	198±21	232±26	231±23	289±26	294±24
Gr_3	SS	112±12	141±14	164±13	189±13	209±17	240±16	261±16	276±16
Gr_4	MC	109±10	143±14	166±15	186±16	204±17	236±16	267±13	273±18
Gr_5	AA	113±12	146±12	168±16	183±11	201±17	240±15	269±116	286±19
Gr_6	RA	108±9	139±13	163±13	182±13	202±13	239±16	269±17	288±16
Gr_7	SS+DMBA	110±12	141±12	162±15	194±21	226±24	246±21	272±21	278±24
Gr_8	MC+DMBA	113±13	143±11	159±17	189±22	231±27	239±23	269±23	264±23
Gr_9	AA+DMBA	108±12	140±12	168±18	192±19	218±20	227±19	229±21	258±24
Gr_{10}	RA+DMBA	114±10	138±12	161±12	186±13	216±18	219±21	249±24	282±21
Gr_{11}	SS+MC	112±13	138±14	167±16	187±11	207±16	241±13	261±14	282±19
Gr_{12}	SS+AA	114±13	140±14	163±14	182±15	201±17	241±16	261±17	279±18
Gr_{13}	SS+RA	111±12	139±15	161±13	180±17	203±13	240±17	263±16	274±18
Gr_{14}	MC+AA	109±10	141±14	165±16	181±17	208±15	242±16	263±16	282±17
Gr_{15}	MC+RA	113±12	142±15	163±17	182±16	201±16	241±17	263±18	279±16
Gr_{16}	AA+RA	117±13	141±13	166±13	181±16	199±17	238±17	263±16	282±20
Gr_{17}	SS+MC+DMBA	116±12	140±12	159±17	196±21	193±21	227±19	268±26	273±19
Gr_{18}	SS+AA+DMBA	114±11	138±10	163±14	187±24	196±19	231±22	273±24	281±23
Gr_{19}	SS+RA+DMBA	112±10	139±13	158±16	199±23	216±14	234±23	281±23	291±26
Gr_{20}	MC+AA+DMBA	109±11	138±14	167±14	198±24	224±19	241±21	269±21	279±23
Gr_{21}	MC+RA+DMBA	113±12	141±15	162±17	181±23	220±18	246±26	271±20	281±24
Gr_{22}	AA+RA+DMBA	110±13	137±12	166±10	186±26	227±22	238±21	281±24	291±21
Gr_{23}	SS+MC+AA	108±12	139±11	165±16	183±18	198±17	237±16	261±19	280±18
Gr_{24}	SS+MC+RA	109±10	141±14	163±19	187±16	202±16	239±19	260±17	278±16
Gr_{25}	MC+AA+RA	113±12	138±13	163±11	186±16	201±14	238±16	262±18	280±17
Gr_{26}	AA+RA+SS	114±13	140±13	162±12	182±16	209±17	239±16	261±18	281±16
Gr_{27}	SS+MC+AA+DMBA	112±10	137±12	160±18	181±23	218±23	230±24	259±21	271±18
Gr_{28}	SS+MC+RA+DMBA	114±12	139±11	159±16	192±21	221±19	227±23	258±23	287±23
Gr_{29}	MC+AA+RA+DMBA	111±10	140±14	163±12	188±24	207±23	222±21	262±26	274±24
Gr_{30}	AA+RA+SS+DMBA	113±12	139±15	160±17	193±23	211±22	236±23	260±24	273±22
Gr_{31}	SS+MC+AA+RA	113±14	141±12	160±16	186±17	214±18	238±18	259±19	278±18
Gr_{32}	SS+MC+AA+RA+DMBA	112±10	137±14	159±18	182±19	209±17	230±19	248±21	267±19

Abbreviations: SS=sodium selenite; MC=magnesium chloride; AA=ascorbic acid; RA=retinyl acetate.

Table III. Effects of Various Combinations of Modulators on DMBA-induced Mammary Carcinogenesis

Group	Treatment(s)	Animal No.		No. of Animals with tumors	Mean No. of tumors per tumor-bearing animal	Tumor type	
		Initial	Effective			AC	FA
Gr_1	Control (vehicle)	15	15	0/15	—	—	—
Gr_2	DMBA	30	28	28/28	3.82 (2–7)[a]	96	11
Gr_3	SS	15	15	0/15	—	—	—
Gr_4	MC	15	15	0/15	—	—	—
Gr_5	AA	15	15	0/15	—	—	—
Gr_6	RA	15	13	0/13	—	—	—
Gr_7	SS+DMBA	30	29	15/29[b]	2.8 (1–5)	38	4
Gr_8	MC+DMBA	30	28	13/28[b]	2.77 (1–6)	31	5
Gr_9	AA+DMBA	30	28	16/28[b]	2.63 (1–5)	36	6
Gr_{10}	RA+DMBA	30	27	13/27[b]	2.85 (1–5)	32	5
Gr_{11}	SS+MC	15	14	0/14	—	—	—
Gr_{12}	SS+AA	15	15	0/15	—	—	—
Gr_{13}	SS+RA	15	14	0/14	—	—	—
Gr_{14}	MC+AA	15	14	0/14	—	—	—
Gr_{15}	MC+RA	15	14	0/14	—	—	—
Gr_{16}	AA+RA	15	13	0/13	—	—	—
Gr_{17}	SS+MC+DMBA	30	27	8/27[b]	2.5 (1–5)	17	3
Gr_{18}	SS+AA+DMBA	30	29	9/29[b]	2.56 (1–4)	20	3
Gr_{19}	SS+RA+DMBA	30	27	8/27[b]	2.38 (1–3)	17	2
Gr_{20}	MC+AA+DMBA	30	27	7/27[b]	2.29 (1–4)	13	3
Gr_{21}	MC+RA+DMBA	30	26	8/26[b]	1.88 (1–3)	13	2
Gr_{22}	AA+RA+DMBA	30	26	9/26[b]	2.11 (1–3)	17	2
Gr_{23}	SS+MC+AA	15	14	0/14	—	—	—
Gr_{24}	SS+MC+RA	15	13	0/13	—	—	—
Gr_{25}	MC+AA+RA	15	13	0/13	—	—	—
Gr_{26}	AA+RA+SS	15	14	0/14	—	—	—
Gr_{27}	SS+MC+AA+DMBA	30	27	6/27[b]	2.0 (1–3)	10	2
Gr_{28}	SS+MC+RA+DMBA	30	26	5/26[b]	1.8 (1–3)	8	1
Gr_{29}	MC+AA+RA+DMBA	30	25	4/25[b]	2.0 (1–3)	8	0
Gr_{30}	AA+RA+SS+DMBA	30	26	6/26[b]	1.67 (1–3)	9	1
Gr_{31}	SS+MC+AA+RA	15	13	0/13	—	—	—
Gr_{32}	SS+MC+AA+RA+DMBA	30	25	3/25[b]	1.67 (1–2)	5	0

Abbreviations: AC=adenocarcinoma; FA=fibroadenoma; others as in Table II.
a) Range of tumor number. *b*) $P<0.001$.

percentage of animals with mammary tumors in different groups.

The control animals (Gr_1) did not develop any tumors during the observation period. Similarly, animals treated only with any one modulator (Gr_3, Gr_4, Gr_5 and Gr_6), combinations of two modulators (Gr_{11}, Gr_{12}, Gr_{13}, Gr_{14}, Gr_{15} and Gr_{16}), combinations of three modulators (Gr_{23}, Gr_{24}, Gr_{25} and Gr_{26}) or all four modulators (Gr_{31}) also did not yield any mammary tumors. On the other hand, animals exposed to DMBA alone (Gr_2) developed tumors in 100% of the cases and the mean tumor number per tumor-bearing animal was 3.82. When DMBA was administered to animals being treated with selenium (Gr_7), magnesium (Gr_8), ascorbic acid (Gr_9) or retinyl acetate (Gr_{10}), the mammary tumor incidences in these groups were reduced to 51.7%, 46.6%, 57.1% and 48.1%, respectively, and the mean tumor numbers per tumor-bearing animal in these four groups (Gr_7–Gr_{10}) were reduced to 2.8, 2.77, 2.63 and 2.85, respectively. Likewise, when DMBA was given to the rats being treated with selenium+magnesium (Gr_{17}), selenium+ascorbic acid (Gr_{18}), selenium+retinyl acetate (Gr_{19}), magnesium+ascorbic acid (Gr_{20}), magnesium+retinyl acetate (Gr_{21}) or ascorbic acid+retinyl acetate (Gr_{22}), the mammary tumor incidences were further reduced in these groups to 29.5%, 31%, 29.6%, 25.9%, 30.8% and

Table IV. DMBA-induced Mammary Tumor Incidences at Different Times during the Observation Period

Group	Treatment(s)	Mammary tumor incidence at different ages (weeks)												
		10	12	14	16	18	20	22	24	26	28	30	32	34
Gr_2	DMBA	0/28[a]	3/28	6/28	9/28	13/28	16/28	17/28	21/28	22/28	24/28	26/27[c]	26/27	27/27
Gr_7	SS+DMBA	0/29	1/29	2/29	4/29	4/29	7/29	7/29	10/29	12/29	13/29	15/29	15/29	15/29
Gr_8	MC+DMBA	0/28	0/28	2/28	4/28	4/28	6/28	8/28	8/28	8/28	9/28	9/28	12/28	13/28
Gr_9	AA+DMBA	0/29	1/28	1/28	3/28	4/28	7/28	9/28	9/28	10/28	12/28	15/28	15/28	16/28
Gr_{10}	RA+DMBA	0/28	1/27	2/27	4/27	4/27	5/27	7/27	10/27	10/27	12/27	12/27	13/27	13/27
Gr_{17}	SS+MC+DMBA	0/28	0/27	1/27	2/27	3/27	3/27	4/27	6/27	7/27	7/27	8/27	8/27	8/27
Gr_{18}	SS+AA+DMBA	0/29	1/29	2/29	2/29	3/29	3/29	4/29	4/28[b]	6/28	7/28	7/28	8/28	9/28
Gr_{19}	SS+RA+DMBA	0/28	0/27	1/27	2/27	3/27	3/27	5/27	6/27	6/27	6/27	8/27	8/27	8/27
Gr_{20}	MC+AA+DMBA	0/27	0/27	2/27	2/27	2/27	3/27	4/27	5/27	5/27	7/27	7/27	7/27	7/27
Gr_{21}	MC+RA+DMBA	0/26	0/26	1/26	2/26	2/26	3/26	5/26	5/26	6/26	8/26	8/26	8/26	8/26
Gr_{22}	AA+RA+DMBA	0/28	0/26	2/26	2/26	3/26	4/26	6/26	7/26	7/26	9/26	9/26	9/26	9/26
Gr_{27}	SS+MC+AA+DMBA	0/28	0/27	0/27	0/27	2/27	3/27	3/27	4/27	4/27	6/27	6/27	6/27	6/27
Gr_{28}	SS+MC+RA+DMBA	0/27	0/26	0/26	1/26	2/26	2/26	3/26	3/26	4/26	4/26	5/26	5/26	5/26
Gr_{29}	MC+AA+RA+DMBA	0/27	0/25	0/25	1/25	1/25	2/25	2/25	2/25	3/25	3/25	4/25	4/25	4/25
Gr_{30}	AA+RA+SS+DMBA	0/28	0/26	0/26	1/26	2/26	2/26	3/26	4/26	4/26	5/26	6/26	6/26	6/26
Gr_{32}	SS+MC+AA+RA+DMBA	0/27	0/25	0/25	0/25	1/25	1/25	2/25	2/25	2/25	3/25	3/24[b]	3/24	3/24

Abbreviations: as in Table II.

a) The numerators indicate the number of rats with tumors and the denominators indicate the number of effective rats in the group.

b) One rat without mammary tumor died.

c) One rat with mammary tumors died.

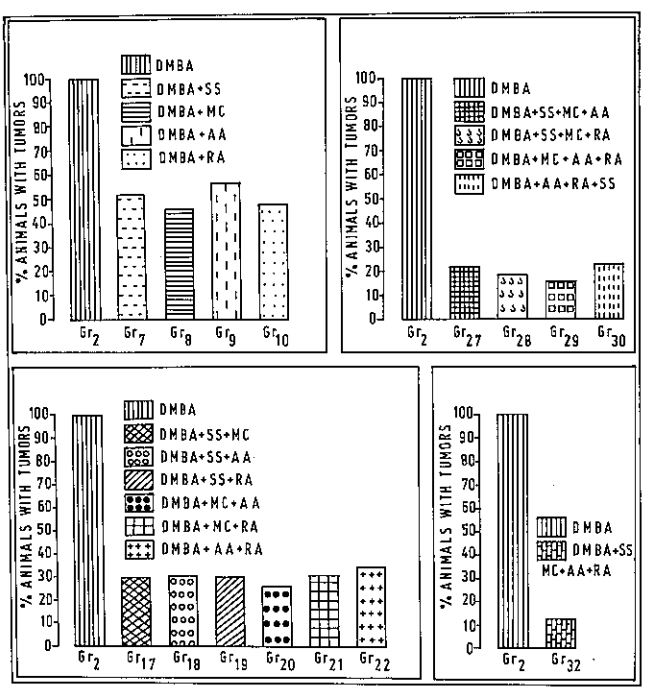

Fig. 2. Influence of modulators on the incidence (%) of mammary tumors induced by DMBA in rats. Abbreviations as in Table II.

34.6%, respectively. The mean tumor numbers per tumor-bearing animal of these six groups (Gr_{17}–Gr_{22}) declined to 2.5, 2.56, 2.38, 2.29, 1.88 and 2.11, respectively. Following administration of DMBA to the animals being treated with three different modulators, i.e., selenium+magnesium+ascorbic acid (Gr_{27}), selenium+magnesium+retinyl acetate (Gr_{28}), magnesium+ascorbic acid+retinyl acetate (Gr_{29}) and ascorbic acid+retinyl acetate+selenium (Gr_{30}), the tumor incidences declined to 22.2%, 19.2%, 16% and 23.1% and the mean tumor numbers per tumor-bearing animal were 2, 1.8, 2 and 1.67, respectively. When DMBA was administered to the animals being concurrently exposed to all four modulators (selenium + magnesium + ascorbic acid + retinyl acetate) (Gr_{32}), the tumor incidence was reduced to 12% and the mean tumor number per tumor-bearing animal was 1.67.

In all tumor-yielding groups both adenocarcinomas and fibroadenomas were present but the former were greater in number in all such groups.

DISCUSSION

The present study not only confirms earlier studies on the chemopreventive actions of selenium, magnesium, ascorbic acid, and retinyl acetate,[5-16] but also demon-

strates that concomitant administration, on a long-term basis, of different combinations of these modulators to the rats resulted in substantial inhibition of DMBA-induced mammary carcinogenesis.

Multiple intubations of small doses of DMBA, instead of a single large dose, were preferred in the present study in order to increase the likelihood of inhibitory actions, if any, of different modulators on the initiational events elicited by DMBA in the mammary epithelium. DMBA, when given alone by this schedule in the present study, could produce mammary tumors in 100% of the rats by 34 weeks of age. When DMBA was given to the animals that were being chronically treated with individual modulators, the mammary tumor incidence was reduced to 46 to 57% depending upon the modulator. Concomitant treatment with any two modulators resulted in the occurrence of DMBA-induced mammary tumors in about 25 to 34% of animals, depending upon the combinations. Likewise when combinations of three modulators were given concurrently the incidence of DMBA-induced mammary tumors was only about 16 to 23% of animals. When all four modulators were given concurrently, the mammary tumor incidence due to DMBA was further reduced to 12%.

In the present study the doses of modulators were decreased in steps during the treatment period. The purpose behind this reduction in the doses was to avoid cumulative toxic effects, if any. It should be noted here that whenever ascorbic acid and sodium selenite were added to the same drinking water, the latter compound was reduced to elemental selenium, which remained suspended in the water.

A number of recent studies have already demonstrated that selenium supplementation to the diet or water can inhibit mammary carcinogenesis induced by chemical carcinogens.[5, 6, 7, 8, 17, 23] Selenium is an essential trace element for mammalian species and is a potent antioxidant. The mechanism by which selenium accomplishes chemoprevention of cancer is presently unclear. Many workers have shown that selenium can be effective as an anticarcinogenic agent in the promotional phase of carcinogenesis elicited by those carcinogens which do not require biotransformation and activation.[24] It is suggested that the selenium, acting through the increase of glutathione peroxidase activity, which may prevent the formation of oxygen free radicals, interferes with the initiation of carcinogenesis.[25, 26] Selenium supplementation increased the activity of glutathione-*S*-transferase involved in the detoxification of carcinogens.[27, 28] It is also known to inhibit carcinogen-DNA adduct formation, thus exerting a preventive action against carcinogenesis.[23, 29, 30]

The anticarcinogenic influence of magnesium has been demonstrated by certain animal studies.[10, 11] The epidemiological evidence in support of anticarcinogenic action of magnesium has been reviewed by Blondell.[12] How magnesium inhibits chemical carcinogenesis is not known. Magnesium ion, being electrophilic, may compete with carcinogens for binding sites of DNA. It was also suggested that intracellular deficiences in magnesium contribute to cancer.[31] Studies by Bowen-Pope *et al.*[32] indicate that magnesium regulates the onset of DNA synthesis. As magnesium is known to have the ability to metabolize benzo[*a*]pyrene into a harmless form,[12] it is possible that it had a similar effect on DMBA in the present study.

Retinoids have been shown to possess anticarcinogenic activity when administered during either the initiational phase[33] or promotional phase[17, 34, 35] of chemically induced mammary carcinogenesis. Retinyl acetate has been shown to exert an antiproliferative effect and to inhibit significantly ductal branching and end-bud proliferation in the mammary glands of rats.[34] Also, it has been shown that retinyl acetate inhibits chemical carcinogen-induced increases in mammary gland DNA synthesis,[36] and the induction by carcinogen of terminal ductal hyperplasia, a putative precancerous lesion.[33] McCormick and Moon[37] showed that retinoids are still effective cancer chemopreventive agents even if the administration is delayed for some time after the carcinogenic insult. In the present study, retinyl acetate was given during the initiation and promotion phases of carcinogenesis, and hence its inhibitory action might have occurred at either or both phases. Recent studies suggested that retinoids act, at least in part, by modulating protein phosphorylation/dephosphorylation reactions in cells, possibly through their action on the activities of specific protein kinases.[38] Since protein phosphorylation is important in cellular regulation, and cAMP-dependent protein kinase is a major regulatory element in cells, it is reasonable to believe that retinoids, which influence protein kinase activity, could profoundly alter cellular transformation.

Chronic administration of ascorbic acid during the initiational and promotional phases of DMBA-induced mammary carcinogenesis in the present study inhibited the tumor incidence appreciably. Earlier studies on the influence of ascorbic acid on the process of chemical carcinogenesis have given equivocal results. Some studies indicated the anticarcinogenic action of ascorbic acid and its derivatives[4, 13, 14] whereas Migliozzi[39] observed that ascorbate deprivation retarded tumor growth and resupplementation enhanced it.

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