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Synergistic Effect of Radiation on Colon Carcinogenesis Induced by Methylazoxymethanol Acetate in ACI/N Rats

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The effect on colon and liver carcinogenicity in rats of a single X-irradiation exposure given either before or after methylazoxymethanol (MAM) acetate was studied in ACI/N rats of both sexes. A single dose of X-irradiation (3 Gy) was administered either 3 months before or after three weekly s.c. injections of MAM acetate (25 mg/kg body weight). At 365 days after the start, the incidence and multiplicity of MAM acetate-induced intestinal tumors were enhanced by X-irradiation either prior to or after the MAM acetate treatment. In addition, X-irradiation before MAM acetate increased the incidence of hepatocellular foci in either sex. In females, X-irradiation either before or after MAM acetate exposure decreased intestinal tumorigenesis. These findings suggest an apparent synergism of these agents in intestinal carcinogenesis of male rats.

Key words: Synergistic effect — X-irradiation — MAM acetate — Intestinal carcinogenesis — Rat

Recently, combination therapy involving X-irradiation, anticancer agents, and occasionally hyperthermia or biological response modifiers has been shown to exert significant therapeutic effects against malignant neoplasms. However, X-irradiation and several anticancer agents, particularly alkylating agents, are potential carcinogens.[1-3] Moreover, second primary tumors could appear after complete response of primary malignant neoplasms.[4] Epidemiological studies indicated a high incidence of neoplasms in several organs including digestive organs in atomic bomb survivors.[5,6] Similarly X-irradiation was reported to induce tumors preferentially in some organs in rodents.[7] Thus, it is possible that chemical carcinogens and X-irradiation could interact, but the potential for synergism[8] between carcinogens and radiation does not appear to have been well studied. Although several investigators have tried to explore the combined effects of X-irradiation and a chemical carcinogen, the results are basically contradictory: some found a synergistic or summative effect and others, no significant effect (see Ref. 9). These studies have employed different types of chemical carcinogens and radiation (moderate to high radiation exposures and single or multiple radiation exposures). The relative timing of radiation exposures and carcinogen treatments is also one of the factors that appears to affect the incidences and latent periods of the tumors. Previously we demonstrated a synergistic effect of X-irradiation on N-2-fluorenylacetamide-induced rat liver carcinogenesis.[10] More recently, an enhancing effect of X-irradiation on N-methyl-N-nitrosourea-induced multi-organ carcinogenesis in rats was found by us.[11] It is important to investigate the modifying effect of X-irradiation on chemical carcinogenesis, since anticncer agents that mostly have carcinogenic potency are used together with X-irradiation therapeutically, and also potent carcinogens and radiation are present together in the environment.

In the present study, the modifying effect of whole-body X-irradiation either before or after methylazoxymethanol (MAM) acetate exposure on intestinal and liver carcinogenesis was examined using ACI/N rats of both sexes.

MATERIALS AND METHODS

Animals and diet A total of 116 inbred ACI/N rats of both sexes (61 males and 55 females), which had been maintained in our laboratory, were used. At 5 weeks of age, these rats were transferred to the holding room, and randomized into experimental and control groups. Rats were housed three or four to a wire cage. The holding room was maintained at 23±2°C and 50±10% humidity, with a 12 h light-dark cycle. Pellet CE-2 (CLEA Japan, Inc., Tokyo) was used as a basal diet. All rats were provided with diet and tap water *ad libitum*.

Chemical and X-irradiation MAM acetate was purchased from Sigma Chemical Co., St Louis, MO. Animals were treated with whole-body X-irradiation of 3 Gy. The conditions of X-irradiation, based on those used in our earlier studies,[10,11] were as follows: 200 kVp; filter, 0.5 mm Cu+0.5 mm Al; focus-to-animal distance, 97.5 cm; tube current, 20 mA; half-value thickness, 1.2 mm Cu; and dose rate, 0.254 Gy/min.

Experimental procedure Animals were divided into six groups. Starting at 8 weeks of age, groups 1 (10 males and 9 females), 2 (12 males and 7 females), and 3 (11

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males and 9 females) were given i.p. injections of MAM acetate (25 mg/kg body weight) once a week for 3 weeks. Rats in groups 2 and 3 were X-irradiated at 6 and 18 weeks of age, respectively. Similarly, animals in groups 4 (8 males and 10 females) and 5 (10 males and 11 females) received X-irradiation alone. Group 6 (10 males and 9 females) served as an untreated control.

Animals were weighed weekly until they reached 22 weeks of age, and then every 4 weeks. The experiment was terminated 365 days after the start. No animals died during the experiment and complete necropsies were performed on all animals at the termination of the experiment. All organs, especially the intestine, were carefully inspected grossly when opened and all abnormal lesions were examined histologically. All tissues were processed for histologic examination by conventional methods and stained with hematoxylin and eosin for histological diagnosis. Intestinal tumors were diagnosed according to the criteria described by Ward.[12] Renal tumors were diagnosed according to the criteria described by Hard and Butler.[13]

Statistics Statistical analysis of tumor incidence and multiplicity was done by using the chi-square test, Fisher's exact probability test and Student's *t* test.

RESULTS

General observations Mean body and liver weights in each group at the termination of the experiment are indicated in Table I. In males, the mean body weight in group 5 was significantly larger than that in group 6 ($P<0.05$). The mean liver weights in groups 2 and 3 were significantly smaller than those in groups 4 and 5, respectively ($P<0.01$). The mean relative liver weight (g/100 g body weight) in group 3 was significantly greater than that in group 1 or 5 ($P<0.05$ or $P<0.01$). In females, mean body, liver and relative liver weights in groups 1–5, except mean body weight in group 5, were lower than those in group 6, but there were no significant differences between groups.

Intestinal tumor incidence and multiplicity Incidences of intestinal tumors in male and female rats of each group are shown in Table II. Histologic examination revealed that neoplasms developed in the intestine were adenomas or adenocarcinomas. In males, combined treatment with MAM acetate and X-irradiation increased the incidence of intestinal neoplasms compared to that of rats given MAM acetate alone. The incidences of adenoma and adenocarcinoma of the small intestine in group 3 were significantly greater than those in group 1 ($P<0.01$ and $P<0.05$). The combined incidence of adenoma and adenocarcinoma of the small intestine in group 3 was also significantly larger than that of group 1 ($P<0.01$). The incidence of colon adenocarcinoma in group 3 was significantly greater than that of group 1 ($P<0.01$). In females, combined treatment with MAM acetate and X-irradiation reduced the incidence of intestinal tumors when compared to group 1, but the effect was without statistical significance. There were no intestinal tumors in males and females of groups 4–6. Multiplicities of intestinal tumors (number of tumors/rat) are summarized in Table III. In male rats, the multiplicities of small intestinal and colon adenocarcinomas in group 3 were significantly greater than in group 1 ($P<0.01$). The combined incidence of adenoma and adenocarcinoma in the small intestine or colon in group 3 was also signifi-

Table I. Mean Body Weight, Liver Weight and Relative Liver Weight at the End of the Study

Group no.	Treatment	No. of rats examined	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g/100 g body wt.)
1	MAM acetate	♂ 10	246±39[a]	9.8±2.2	3.97±0.62
		♀ 9	169±20	8.0±1.4	4.71±0.43
2	R[b]/MAM acetate	♂ 12	232±19	8.9±1.1[c]	3.83±0.37
		♀ 7	179±17	7.3±1.1	4.06±0.36
3	MAM acetate/R	♂ 11	218±32	10.4±1.8[c]	4.81±0.78[d, e]
		♀ 9	176±13	7.0±1.1	3.95±0.42[e]
4	R/−	♂ 8	266±25	11.1±1.5	4.23±0.70
		♀ 10	175±15	6.8±0.8	3.87±0.23
5	−/R	♂ 10	311±24[d]	12.2±1.0	3.92±0.25
		♀ 11	198±8	8.0±0.9	4.03±0.45
6	No treatment	♂ 10	273±32	11.1±1.7	4.05±0.32
		♀ 9	188±9	8.2±0.8	4.36±0.36

a) Mean±SD.
b) R indicates X-irradiation.
c–e) Significantly different from appropriate controls by Student's *t* test (*c*, $P<0.01$; *d*, $P<0.05$; *e*, $P<0.01$).

Table II. Incidence of Intestinal Tumors

Group no.	Treatment	No. of rats examined	No. of rats with tumors (%) at:					
			Small intestine			Colon		
			Total	AD[a]	ADC	Total	AD	ADC
1	MAM acetate	♂ 10	1 (10)	0	1 (10)	7 (70)	4 (40)	5 (50)
		♀ 9	2 (22)	1 (11)	1 (11)	8 (89)	6 (67)	4 (44)
2	R/MAM acetate	♂ 12	2 (17)	2 (17)	1 (8)	10 (83)	6 (50)	8 (67)
		♀ 7	0	0	0	4 (57)	3 (37)	3 (37)
3	MAM acetate/R	♂ 11	9 (82)[b]	7 (64)[b]	7 (64)[c]	11 (100)	6 (55)	11 (100)[b]
		♀ 9	2 (22)	0	2 (22)	4 (44)	4 (44)	3 (33)

a) AD, adenoma; ADC, adenocarcinoma; R, X-irradiation.
b, c) Significantly different from group 1 by Fisher's exact probability test (*b*, $P<0.01$; *c*, $P<0.05$).

Table III. Multiplicity (No. of Tumors/Rat) of Intestinal Tumors

Group no.	Treatment (no. of rats examined)	Multiplicity of tumors at:					
		Small intestine			Colon		
		Total	AD[a]	ADC	Total	AD	ADC
1	MAM acetate (♂ 10)	0.10 ±0.30	0	0.10 ±0.30	1.40 ±1.62	0.70 ±1.00	0.70 ±0.78
	(♀ 9)	0.22 ±0.42	0.11 ±0.31	0.11 ±0.31	1.44 ±0.96	1.00 ±0.94	0.44 ±0.50
2	R/MAM acetate (♂ 12)	0.50 ±1.38	0.25 ±0.60	0.25 ±0.83	2.42 ±1.75	0.67 ±0.75	1.75 ±1.42
	(♀ 7)	0	0	0	1.00 ±0.93	0.43 ±0.49	0.57 ±0.73
3	MAM acetate/R (♂ 11)	2.55[b] ±1.78	1.09 ±1.00	1.45[c] ±1.37	6.00[c] ±4.82	1.64 ±2.01	4.36[c] ±3.17
	(♀ 9)	0.22 ±0.42	0 0	0.22 ±0.42	0.89 ±1.10	0.56 ±0.68	0.33 ±0.47

a) AD, adenoma; ADC, adenocarcinoma; R, X-irradiation.
b, c) Significantly different from group 1 by Studen's *t* test (*b*, $P<0.001$; *c*, $P<0.01$).

Table IV. Incidence of Liver Cell Foci and Neoplasms

Group no.	Treatment	No. of rats examined	No. of rats (%) with	
			Liver cell foci	Liver cell adenomas
1	MAM acetate	♂ 10	4 (40)[a]	0
		♀ 9	2 (22)	0
2	R/MAM acetate	♂ 12	12 (100)[b, c]	2 (17)
		♀ 7	6 (86)[d, e]	0
3	MAM acetate/R	♂ 11	11 (100)[f]	0
		♀ 9	8 (89)[f]	0
4	R/−	♂ 8	1 (13)	0
		♀ 10	1 (10)	0
5	−/R	♂ 10	9 (90)[g]	0
		♀ 11	8 (73)[h]	0
6	No treatment	♂ 10	0	0
		♀ 9	0	0

a) $P<0.05$ vs. group 6. *b*) $P<0.01$ vs. group 1. *c*) $P<0.001$ vs. group 4.
d) $P<0.05$ vs. group 1. *e*) $P<0.01$ vs. group 4. *f*) $P<0.01$ vs. group 1.
g) $P<0.001$ vs. group 6. *h*) $P<0.01$ vs. group 6.

cantly larger than that of group 1 ($P<0.001$ or $P<0.05$). In females, animals treated with MAM acetate and X-irradiation had smaller multiplicity of intestinal tumors than did group 1, but no significant differences between the groups were found.

Tumors and preneoplastic lesions in other organs Besides intestinal tumors, nephroblastomas (nephroblastic or mesenchymal type) developed in females (3 of 9 rats in group 1, 2 of 7 rats in group 2, and 2 of 9 rats in group 3) and liver cell adenomas in males (2 of 12 rats in group 2). However, significant differences in incidence between groups were seen. As shown in Table IV, liver cell foci, which are regarded as preneoplastic lesions for liver cell neoplasms, were found in males and females of all groups except group 6. In group 1, the foci developed in 4 of 10 males (40%) and 2 of 9 females (22%). All males and 6 of 7 females (86%) had hepatocellular foci in group 2. In group 3, all males and 8 of 9 females (89%) had foci. Foci were present in 1 of 8 males (13%) and 1 of 10 females (10%) in group 4. In group 5, foci were found in 9 of 10 males (90%) and 8 of 11 females (73%). No foci were seen in rats of group 6. The incidences of foci in males and females of group 2 were significantly higher than those in group 1 ($P<0.01$ for males and $P<0.05$ for females). Also, the frequencies of foci in rats of group 3 were significantly greater than those in rats of group 1 ($P<0.01$ for males and $P<0.01$ for females) and those in group 4 ($P<0.001$ for males and $P<0.01$ for females). However, the frequencies of foci in either sex of group 3 were comparable to those in group 5. The incidences of foci in group 5 were significantly higher than those in group 6 ($P<0.001$ for males and $P<0.01$ for females).

DISCUSSION

The results in the present study demonstrated that when X-irradiation is combined with a carcinogen, MAM acetate, either before or after the carcinogen treatment, the incidences of small intestinal and colon tumors were increased compared to those obtained with each agent alone, at least in male rats. Also, X-irradiation before i.p. injections of MAM acetate increased the development of hepatocellular foci in both sexes.

Previously, combined effects of X-irradiation and chemical carcinogens in tumor induction have been observed in a variety of rodent systems, but the findings were contradictory: synergy, additivity, antagonism or lack of effect.[9] Such results may be due to the different types of carcinogens and radiation, and the dose or timing of radiation.[14] Synergistic effects of X-irradiation on chemical carcinogenesis were observed in 7,12-dimethylbenz[*a*]anthracene-induced carcinogenesis in hamster cheek pouch[15] and lingua.[16] In our previous investigations, in which X-irradiation was applied after the carcinogen exposure in rats, synergistic or enhancing effects of X-irradiation on N-2-fluorenylacetamide-induced liver carcinogenesis[10] and on N-methyl-N-nitrosourea-induced multi-organ tumorigenesis[11] were apparent. Recently, Sharp and Crouse[17] reported a synergism between X-irradiation and 1,2-dimethylhydrazine (DMH) in the development of colonic neoplasms in rats. Their study involved two experiments in which rats received a single (9 Gy) or triple abdominal radiation exposure (9 Gy, 3 times), followed by a single or triple DMH treatment, and they found that single or triple radiation exposure before single or triple DMH injection clearly enhanced DMH-induced colon carcinogenesis. The results in the current study are consistent with those in the other studies[10, 11, 16–21] and confirm the report by Sharp and Crouse.[17] In the latter study,[17] a single abdominal radiation exposure alone induced a colon tumor in one of 14 male F344 rats (7.1%), as did triple radiation exposure alone. In the present study, single X-irradiation (3 Gy) either before or after MAM acetate treatment did enhance the development of intestinal tumors, and X-irradiation alone did not produce intestinal tumors in male ACI/N rats. The absence of intestinal tumors in rats treated with X-irradiation alone may be due to the low dose of X-irradiation and/or low susceptibility to intestinal tumors in this strain. In females, X-irradiation did not enhance but rather reduced the intestinal neoplasms induced by MAM acetate. This sex difference in the interactions between X-irradiation and the carcinogen is possibly in part due to hormonal differences.[22, 23]

In the present study, X-irradiation before MAM acetate administration increased the occurrence of liver cell foci in either sex. These results are in agreement with those in our previous study[10] and those reported by others.[24–26] X-irradiation alone in the current study could produce the foci in either sex and unexpectedly, rats irradiated at 18 weeks of age had a higher incidence of foci than did those at 6 weeks of age. This is in contrast to the report by Kitagawa *et al.*[27] However, in other organs such as the breast, female SD rats irradiated at older age had higher tumor yields compared with those at younger age.[28]

Synergism in chemical carcinogenesis occurs when the effect of two or more substances acting together exceeds the sum of their effects when acting separately.[8, 29] Since complete carcinogens act as both initiators and promoters,[30] care is needed to avoid confusion of the interactions obtained. Many tests of carcinogenic synergism in experimental animals employed high dose(s) of one or both of the two carcinogens, but carcinogenesis in humans generally occurs in the low-dose response range. In this context, Reif[8] has proposed that doses that

produce a tumor incidence of approximately 10–20% when the carcinogens are applied singly may be ideal. In the present study, the dose of MAM acetate used for induction of intestinal tumors was that considered adequate for initiation, and resulted in 70% or 89% incidence of colonic neoplasms. Williams and Furuya[31] previously proposed a reverse sequence protocol for distinguishing between initiation-promotion and syn-carcinogenesis by sequentially administered chemicals. Our experimental design in this study was consistent with the proposed protocol. A possible mechanism for synergism in the present study involves the relationship between X-irradiation and the carcinogen binding to DNA. X-irradiation might alter quantitatively or qualitatively, through DNA damage or cytokinetic mechanisms, the binding of the ultimate metabolite (MAM) to intestinal mucosal cell DNA and the alkylation of DNA. Epithelial cell proliferation,[32–35] which is increased by X-irradiation or carcinogen exposure, may also play a significant role in the synergism in the present study, as described previously.[11]

Although apparent synergism between X-irradiation and carcinogen exposure is present in this model, the doses of both X-irradiation and carcinogen used in this study are much higher than those which might be encountered in an environmental situation. However, the potent synergy observed here suggests the need for caution when both types of biological attack, which may lead to alteration of cell proliferation in the target organ(s), are present. Since anticancer agents are also carcinogens, there is considerable potential for induction of preneoplastic lesions or neoplasms when such compounds are combined with X-irradiation in the clinical situation.

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