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Influence of Aging on Multi-organ Carcinogenesis in Rats Induced by N-Methyl-N-nitrosourea

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The effects of aging on the multi-organ carcinogenesis induced in rats by N-methyl-N-nitrosourea (MNU), a direct carcinogen which does not need metabolic activation to exert carcinogenicity, were examined in male F344 rats. In the first experiment, rats at 6, 52, and 98 weeks of age were treated with MNU (20 mg/kg body weight, i.p.) twice weekly for 6 weeks and then maintained without any further treatment for 24 weeks in the case of young and middle-aged rats and for 18 weeks in the case of the old rats. In young rats, malignant lymphomas, particularly thymic types, were observed at significantly high incidence. A striking result in the middle-aged rats was the significantly higher incidence of adenocarcinomas in the small intestine than in young or old animals. The induction of proliferative and neoplastic lesions of the large intestine also tended to be increased in middle-aged rats. In addition, epithelial hyperplasia of the tongue, but not the forestomach, occurred at the highest incidence in the middle-aged group. There were no differences in the induction of epithelial lesions in the urinary bladder among the groups. In a second experiment, investigation of DNA synthesis in the tongue, small and large intestines, urinary bladder and lymph nodes did reveal significant increases or tendency for increase in the MNU-treated groups, but without differences with age. In contrast, the thymus of young rats showed significantly increased incorporation of BrdU label after administration of MNU, whereas it was markedly reduced in middle-aged rats. In a third experiment, O^6-methyldeoxyguanine (O^6-medG) DNA adduct formation was immunohistochemically detected in various organs including the thymus, forestomach, and small intestine without any differences with age. Thus, the results demonstrated that while the target organs of MNU are modified by the age of the animals, levels of DNA synthesis and O^6-medG DNA adduct formation in most cases can not explain the observed differences in carcinogenic susceptibility.

Key words: Aging and cancer — Multi-organ carcinogenesis — N-Methyl-N-nitrosourea — O^6-Methyldeoxyguanine — Cell proliferation

There have been many reports of an influence of age of animals upon induction of neoplastic lesions by chemical carcinogens.[1-5] Much evidence has indicated that young animals are more sensitive than old ones,[6-10] although in some cases an age-related increase in susceptibility to chemical carcinogenesis has been reported.[11, 12] Recently we found such an age-related increase in the development of urinary bladder carcinomas in rats of both sexes treated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN[6]), and established that this was not dependent on intake of carcinogen.[13] Since indirect carcinogens such as BBN need metabolic activation to exert their carcinogenicity, this might have played a role. In general, however, the activities of liver microsomal enzymes show age-related decrease, resulting in age-dependent decrease in metabolic activation of carcinogens. Differences in the susceptibility of young and old animals to the carcinogenicity of BBN can therefore not simply be explained in terms of its metabolism, although this is clearly a complicating factor.

In the present study, we examined the effects of aging on multiple-organ carcinogenesis induced in rats by N-metyl-N-nitrosourea (MNU), a direct carcinogen which does not need metabolic activation to exert its effects. Thus, attention could be concentrated on the effects of aging on DNA synthesis and formation of DNA alkylation adducts in target organ cells as possible contributory factors to the observed differences in susceptibility to MNU carcinogenicity.

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[6] The abbreviations used are: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; MNU, N-methyl-N-nitrosourea; ABC, avidin-biotin-peroxidase complex; BrdU, 5-bromo-2′-deoxyuridine; O^6-medG, O^6-methyldeoxyguanine.

MATERIALS AND METHODS

Animals A total of 212 male F344 rats were purchased from Charles River Japan, Inc., Atsugi at 5 weeks of age. The rats were housed five per plastic cage with wood chips for bedding in an animal room with a 12-h light/12-h dark cycle at 22±2°C (SD) and 55±10% relative humidity.

Chemicals MNU was purchased from Sakai Rikagaku Kenkyujyo, Fukui, and dissolved at 20 mg/ml in ice-cold citrate buffer (adjusted to pH 6.0) immediately before use.

Experimental protocols

Experiment 1 One hundred and thirty rats were randomly divided into 4 groups and maintained on basal diet and tap water until the ages of 6, 52 or 98 weeks. Group 1 (thirty animals at 6 weeks of age) was treated with MNU (20 mg/kg body wt., i.p.) twice a week (at the beginning of the week and on the 4th day) for 6 weeks and then maintained on basal diet (Oriental MF; Oriental Yeast Co., Ltd., Tokyo) and tap water without any treatment for 24 weeks. Thirty middle-aged (52-week-old) rats (group 2) were similarly given MNU for 6 weeks and then maintained under ordinary conditions for 24 weeks (up to 82 weeks of age). The 43 surviving rats of group 3 (98-week-old) were given the same 6-week MNU treatment and then kept without any treatment for 18 weeks (up to 122 weeks of age), when all remaining animals were killed, because many had died due to emaciation by that time. In addition, 20 rats of group 4 were maintained without any treatment throughout the experimental period. Food (Oriental MF) and tap water were available *ad libitum* during the experiment. The animals were observed daily for abnormalities and individual body weights were recorded every 2 weeks. Food and water consumptions were measured over a 2-day period before each weighing.

Complete autopsies were performed on rats found dead or killed after they became moribund, as well as at the termination of the study. All organs and tissue were excised, and fixed in 10% phosphate-buffered formalin (pH 7.4) for routine processing and histopathological examination.

Experiment 2 To investigate DNA synthesis in the cells in target organs, 70 rats were randomly divided into 6 groups. Starting at 6 (group 1), 52 (group 3), or 98 (group 5) weeks of age they were treated with MNU (20 mg/kg body wt., i.p.) twice a week (at the beginning of the week and on the 4th day) for 5 weeks. Relevant control groups at the respective ages (groups 2, 4 and 6) were injected intraperitoneally with saline twice a week for the same 5 weeks. Food (Oriental MF) and tap water were available *ad libitum* during the experiment. At the end of weeks 3 and 5 after the start of MNU treatment, 5 rats in each group were injected intraperitoneally with 100 mg/kg body wt. of 5-bromo-2′-deoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO). One hour later the animals were killed under anesthesia. The tongue, forestomach, small intestine, large intestine, thymus, lymph nodes, spleen, and urinary bladder were excised, fixed in 10% phosphate-buffered formalin (pH 7.4) and processed for detection of BrdU incorporation into DNA by immunohistochemical examination of paraffin sections. The avidin-biotin-peroxidase complex (ABC) method with anti-BrdU monoclonal antibody (Becton Dickinson Monoclonal Center, Inc., Rutherford, NJ) was used as previously described.[14] The numbers of cells incorporating BrdU into the DNA per 1000 cells were counted under the light microscope. Labeling indices were expressed as percentage values in the tougue, forestomach, thymus, lymph nodes, spleen and urinary bladder or No./crypt in the small intestine and large intestine.

Experiment 3 To investigate the formation of DNA alkylation adducts, 12 rats were randomly divided into 3 groups. Starting at 6 (group 1), 52 (group 2), or 98 (group 3) weeks of age they were treated with MNU (20 mg/kg body wt., i.p.) twice a week (at the beginning of the week and on the 4th day) for the first 2 weeks. At the beginning of the 3rd week, they received 50 mg/kg body wt., i.p. of MNU and 10 h later were killed under anesthesia. The tongue, forestomach, small intestine, large intestine, thymus, lymph nodes, and urinary bladder were excised, fixed in ice-cold acetone solution and processed for immunohistochemical detection of O^6-methyldeoxyguanine (O^6-medG) adducts. After deparaffinization, the sections were stained immunohistochemically with the ABC method using anti-O^6-medG polyclonal antibodies.[15] As a control for the evaluation of immunohistochemical staining, the first antibody was omitted from some slides. The grading of staining intensity in the nuclei was classified as follows: ±, trace; +, weak; ++, strong.

Data from Experiments 1 and 2 were statistically analyzed by using the one-sided Fisher's exact probability test.

RESULTS

Experiment 1 Most rats in each of the groups had died before the scheduled time of death, because of tumor induction in many organs. Rats in group 3 were killed before the scheduled time because of the development of cachexia with severe anemia and therefore the observation period (18 weeks) after MNU treatment for group 3 was shorter than for groups 1 and 2 (24 weeks). There were no differences in average survival time periods between groups 1 and 2.

Animals that survived for more than 12 weeks after MNU treatment, the time when the first tumor appeared

in the thymus in group 1, were included in the effective numbers for histologic analysis. Proliferative and neoplastic lesions diagnosed in the various organs are summarized in Table I. Malignant lymphomas of thymic type were observed in group 1 at a markedly high incidence. Malignant lymphomas of lymphatic type appeared in significantly higher incidence in group 1 than in group 2. While the incidences of splenic-type malignant lymphomas were significantly higher in groups 3 and 4 than in groups 1 and 2, this appeared to be independent of MNU exposure. However, the total incidence of malignant lymphomas was significantly higher in group 3 (22/39, 56%) than in group 4 (5/18, 28%). In the tongue, the incidence of epithelial hyperplasia was significantly higher in group 2 than in groups 1 and 3. Induction of papillomas and squamous cell carcinoma also tended to be increased in group 2. Incidences of epithelial hyperplasia and papillomas in the forestomach were significantly lower in group 3 than in groups 1 and 2. That of squamous cell carcinoma similarly was lowest in group 3. A striking finding was the significantly higher incidence of adenocarcinomas in the small intestine in group 2 than in groups 1 and 3. The induction of epithelial tumorous lesions in the large intestine also tended to be increased in group 2.

Table I. Incidences of Proliferative and Neoplastic Lesions in Various Organs of Rats Treated with MNU (Experiment 1)

Finding	Age:	Young	Middle	Old	
	Group:	1	2	3	4
	Effective No. of rats:	30	30	39	18
Malignant lymphoma					
Thymic		29 (97)[b]***	0	0	0
Splenic		3 (10)	0	16 (41)**	5 (28)**
Lymphatic		8 (27)**	0	6 (15)	0
Tongue					
Hyperplasia		6 (20)***	22 (73)	3 (8)***	0
Papilloma		0	4 (13)	2 (5)	0
SCC[a]		2 (7)	5 (17)	3 (8)	0
Forestomach					
Hyperplasia		28 (93)	24 (80)	21 (54)*	1 (6)
Papilloma		10 (33)	11 (37)	5 (13)*	0
SCC		9 (30)	7 (23)	3 (8)	0
Small intestine					
Hyperplasia		0	3 (10)	2 (5)	0
Adenoma		3 (10)	0	0	0
Adenocarcinoma		4 (13)**	15 (50)	4 (10)***	0
Large intestine					
Hyperplasia		0	2 (7)	0	0
Adenoma		0	0	0	0
Adenocarcinoma		1 (3)	5 (17)	3 (8)	0
Ear duct					
Hyperplasia		2 (7)	6 (20)	4 (10)	0

a) SCC, squamous cell carcinoma.
b) Numbers in parentheses, percentage values.
Significantly different from group 2 at * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (one-sided Fisher's exact probability test).

As summarized in Table II, there were no differences in the incidences of epithelial lesions of the urinary bladder among the groups, although significant induction was associated with MNU treatment in all cases. All aged rats had Leydig cell tumors in the testis, whereas none of the young rats presented such lesions. Although tumors were observed in groups 1 to 4 in various organs other than those mentioned above, there was no age-related tendency for increase or decrease.

Experiment 2 The results of measurement of DNA synthesis as assessed by incorporation of BrdU into DNA in the tongue, forestomach, small intestine, large intestine, thymus, lymph nodes, and urinary bladder of MNU-treated and control rats of each age are shown in Figs. 1 and 2. BrdU-labeled cells of the forestomach epithelium were significantly more frequent after MNU-treatment at week 5 in group 1, but not at the other ages. Labeling indices of the tongue, small intestine and large intestine epithelia in MNU-treated groups at different ages were not significantly different from the respective control group values, although generally slightly higher.

Table II. Induction of Epithelial Lesions in the Urinary Bladder of Rats Treated with MNU (Experiment 1)

Group	Age	MNU treatment	Effective No. of rats	Epithelial lesions			
				Hyperplasia		Papilloma	Carcinoma
				Simple	PN[a]		
1	Young	+	30	5 (16.7)[b]	1 (3.3)	0	1 (3.3)
2	Middle	+	30	3 (10)	1 (3.3)	1 (3.3)	0
3	Old	+	39	6 (15.4)	1 (2.6)	1 (2.6)	1 (2.6)
4	Old	−	18	0	0	0	0

a) PN, papillary or nodular.
b) Numbers in parentheses, percentage values.

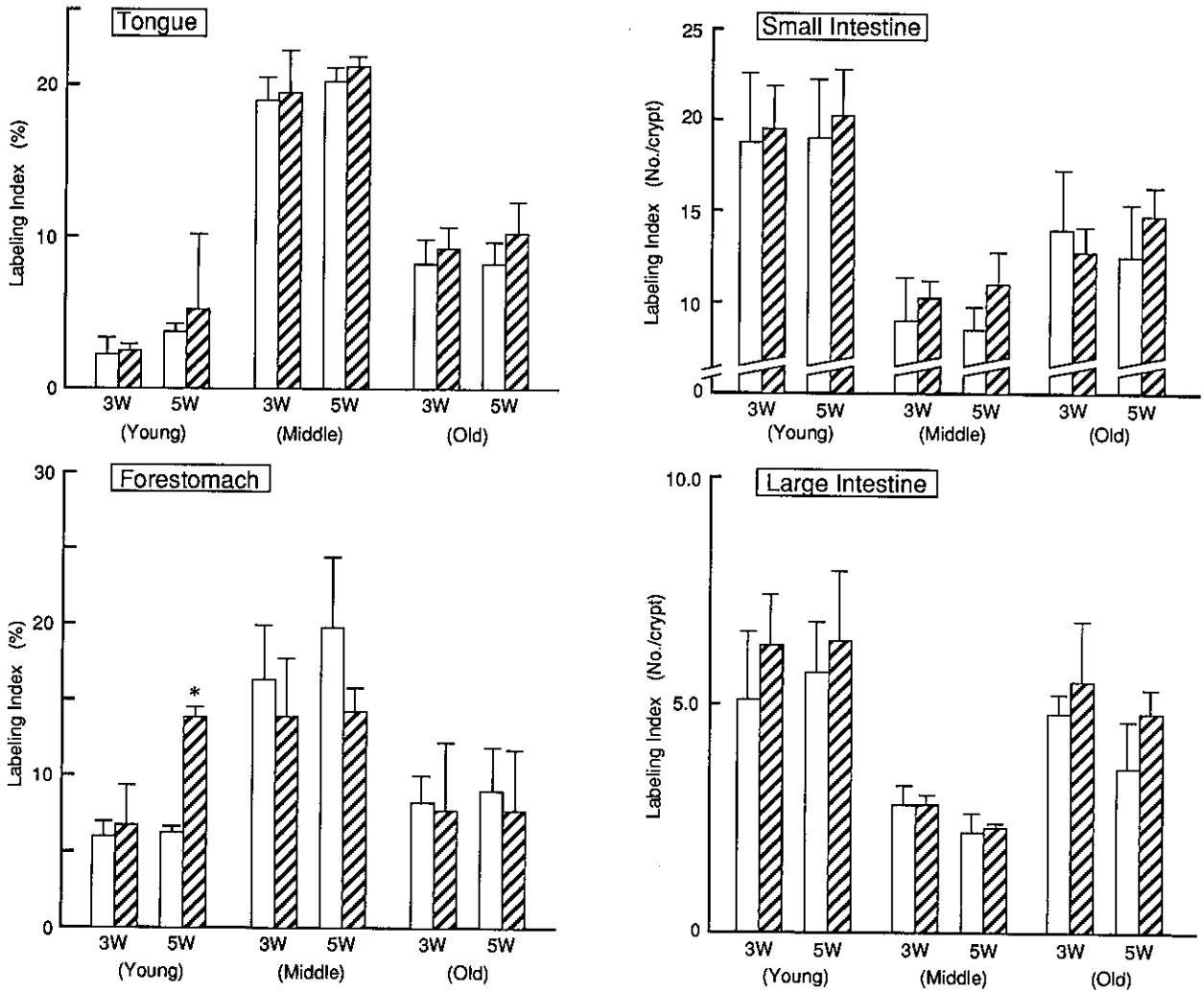

Fig. 1. BrdU labeling indices in the tongue, forestomach, small intestine, and large intestine of rats treated with MNU (Experiment 2). ▨, MNU-treated groups (groups 1, 3 and 5); □ relevant control groups (group 2, 4 and 6).

In the thymus of MNU-treated rats, labeling indices in group 1 at weeks 3 and 5 were significantly increased as compared to group 2, whereas those in group 3 at both time points were significantly decreased as compared to control group 4. Although the labeling index in group 5 at week 3 was significantly higher than in group 6, the value was low. Labeling indices of the lymph nodes in group 1 at weeks 3 and 5 were significantly increased over group 2 values. Labeling indices in the urinary bladder were consistently increased significantly by MNU-treatment (except at week 3 in group 5). Considerable variation in labeling indices in different organs of the controls of different ages was also observed. The highest values were found in the thymus, tongue and forestomach mucosae in middle-aged animals. The same animals, in contrast, had the lowest levels of incorporation in the intestine.

Experiment 3 The results of O^6-medG staining in the thymus, tongue, forestomach, small intestine, large intestine and urinary bladder are summarized in Table III. In the rats treated with MNU, staining of nuclei was not sporadic but rather diffuse throughout the tissue specimens. Moderately strong staining of O^6-medG-positive epithelial cells and lymphocytes was apparent in the thymus of middle-aged and old rats, but not in young rats. Weak O^6-medG staining was found in the squamous epithelium of the tongue in middle-aged and old rats, but again not in young rats, whereas O^6-medG staining in the forestomach epithelium was almost the same in rats of all 3 groups. The epithelia including glands of the small

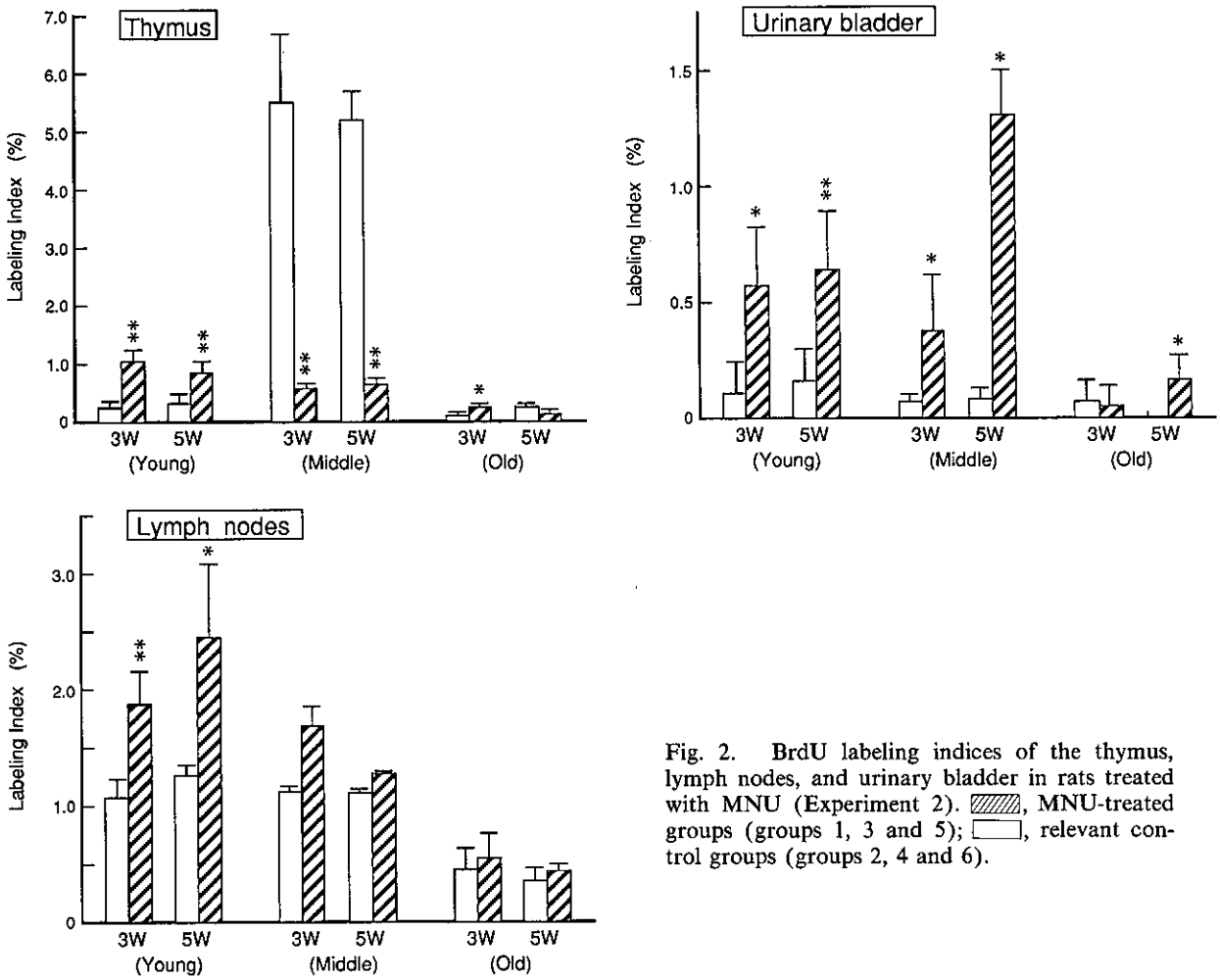

Fig. 2. BrdU labeling indices of the thymus, lymph nodes, and urinary bladder in rats treated with MNU (Experiment 2). ▨, MNU-treated groups (groups 1, 3 and 5); □, relevant control groups (groups 2, 4 and 6).

Table III. Results for Immunohistochemical Staining of O^6-medG in Various Organs of Rats Treated with MNU (Experiment 3)

Age	No. of rats	Thymus				Tongue		Fore-stomach		Small intestine		Large intestine		Urinary bladder	
		Epi.[a]		Lymph.											
		±[b]	+	+	++	±	+	±	+	±	+	±	+	±	+
O^6-MeG															
Young	3	3[c]	0	3	0	3	0	1	2	3	0	2	1	0	3
Middle	3	0	3	0	3	0	3	0	3	3	0	0	3	0	3
Old	3	1	2	1	2	0	3	0	3	0	3	0	3	1	2

a) Epi., epithelial cells; Lymph., lymphocytes.
b) ±, trace; +, weak; ++, strong.
c) Number of rats exhibiting positive staining in nuclei.

intestine in old rats and of the large intestine in middle-aged and old rats showed weakly positive staining, whereas those of the small intestine in young and middle-aged rats showed very weakly positive staining. The staining intensity of the urinary bladder epithelium was almost uniformly weak in young, middle-aged and old rats.

DISCUSSION

In the present study, clear variation in MNU carcinogenesis depending upon the age at which carcinogen exposure occurred was observed. In particular, malignant lymphomas of lymphatic and especially thymic types were found in young rats treated with MNU at a significantly high incidence, whereas splenic-type lesions developed more frequently in old rats, independently of MNU treatment. One of the most interesting results concerns the significantly higher incidences of tongue lesions and adenocarcinomas in the small intestine of middle-aged rats. These, together with the tendency for increased induction of proliferative and neoplastic lesions in the large intestine in group 2 of experiment 1, suggest that the digestive tract of middle-age rats is particularly sensitive to MNU. Epidemiologic data show that although the incidences of cancer in the digestive organs such as in the stomach, intestine, and pancreas do increase with age, the association is not strong at very advanced ages.[16] In contrast, cancers in the urinary bladder and prostate clearly become increasingly prevalent in very old people. Cancers of the hematopoietic, nervous and reproductive systems are found at relatively high incidences even in young groups. Therefore, the results of the present study are generally in agreement with human data, confirming that the age distribution of cancer incidence differs depending on the organs.

The mechanisms underlying modification of MNU multi-organ carcinogenesis by aging in the present study are unclear. In general, it has been considered that various factors may link carcinogenesis with the aging process. As mentioned earlier, aging might influence interaction between carcinogens and cell macromolecules (DNA, RNA, and proteins), because of altered metabolic potential. This could be ruled out, however, in the present experiment using MNU.

It is well known that DNA adducts, generally defined as direct covalent modifications of DNA by chemicals, are intimately associated with the carcinogenic process, especially initiation. O^6-Alkylation in various organs[17, 18] is a form of DNA alkylation which appears particularly important and there is a good correlation between production of O^6-medG and carcinogenicity. For example, it has been reported that formation of O^6-medG in the brain correlated with induction of a high incidence of brain tumors by intravenous injection of alkyl nitrosourea to rats.[17] However, the present findings did not indicate any clear positive link between levels of adducts and sensitivity to tumor induction. Indeed the highest incidence of malignant lymphomas was found in the young group with the least O^6-medG binding. It might be worthwhile conducting a quantitative analysis of O^6-medG binding in target cells to elucidate aging differences. In addition, it has been recently reported that Buf/N rats, which are sensitive to mammary carcinogenesis, and Copenhagen rats, which are resistant, do not differ in the extent of formation or rate of repair of O^6-medG in DNA of mammary epithelial cells treated with MNU.[19] Recently, Ishikawa *et al.*[20] reported that aged mice were less able to repair extensive DNA damage caused by ultraviolet irradiation than young mice, though other researchers found no appreciable age-related decrease in DNA repair capacities.[21, 22] The DNA repair enzyme, O^6-medG-DNA methyltransferase does play a key role in the repair of DNA damage, protecting against carcinogenicity by alkylating agents.[23] Differences in such activity could have been responsible for some variation, and an investigation of the role of O^6-medG-DNA methyltransferase in MNU carcinogenicity and aging differences of rats is clearly required.

Proliferative activities of the MNU target cells might conceivably have caused the differences among the 3 age groups. In chemical carcinogenesis, increased cell proliferation is considered to increase susceptibility to initiation and to account for the effects of nongenotoxic compounds.[24] In the present study, while BrdU labeling indices of the tongue, small and large intestine, urinary bladder and lymph nodes showed significant increases or increasing trends after MNU-treatment there was no consistent relation to tumor induction. In addition, the target cell population may change at different ages and this may have an important role in explaining the different carcinogenic responses. In the thymus, no marked difference between young and middle-aged rats was found in MNU-dependent proliferation, though incorporation of label by control tissue was far higher in the middle-aged rats with lower lymphoma incidence. The fact that MNU-treatment was associated with increase in the young case but decrease in the other suggests, however, fundamental differences between the physiology of the thymus at the two age time points. With regard to the tongue and forestomach squamous cell epithelia, a weak positive correlation between proliferation level and carcinogenic response was found, but with the intestine the correlation was in fact a negative one.

In this study, we could not duplicate the age-related increase in induction of urinary bladder carcinomas found with BBN.[13] While we can not explain the difference in the results, the fact that our experiment used a direct carcinogen, MNU, and the other an indirect carcinogen, BBN, might suggest a role for metabolizing enzymes. Effects of age in multi-organ carcinogenesis in rats were also observed with another indirect carcinogen, 3,2′-dimethyl-4-aminobiphenyl,[25, 26] aged animals again being more susceptible to urinary bladder carcinogenesis and this appearing to correlate with metabolic activation.[26] This could not be the case with MNU, but differ-

ences in the pharmacokinetics of this carcinogen might have played a role in determining carcinogenic susceptibility in the present study.

The Ha-*ras* gene is activated in mammary adenocarcinomas induced by MNU in rats.[27] Therefore, it could be important to know whether the probability of an Ha-*ras* gene mutation differs with aging in various organs treated with MNU. In addition, the question of how suppressor genes might function in the promotion and progression stages of MNU carcinogenesis at different ages requires analysis.

In conclusion, while significant differences in response to MNU carcinogenicity were evident in male F344 rats aged 6, 52 or 98 weeks at the commencement, they did not consistently correlate with either proliferation status or levels of O^6-medG adducts during the initiation stage. The possibility that the post-initiation phases may differ between animals of different ages warrants consideration.

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