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Liver Carcinogenesis by Methyl Carbamate in F344 Rats and Not in B6C3F1 Mice

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Short-term and long-term carcinogenicity of methyl carbamate (MCB) was evaluated in F344 rats and B6C3F1 mice. In experiments lasting 6, 12, and 18 months, MCB was given in water by gavage to groups of 10 male and 10 female rats at 0 or 400 mg/kg body weight, 5 days per week, and to similar groups of mice at 0 or 1,000 mg/kg. At 6 months, MCB induced atypical mitoses, cytologic alterations, cytomegaly, pigmentation, necrosis, and neoplastic nodules of the liver in rats. At 12 and 18 months, carcinomas of the liver were induced by MCB in 80–90% of male rats and in 60–80% of female rats. None was observed in control rats or in mice. In the 2-year studies, MCB was given to groups of 50 male and 50 female rats at 0, 100, or 200 mg/kg and to similar groups of mice at 0, 500, or 1,000 mg/kg, 5 days/week. Chronic focal inflammation, cytologic alteration, hyperplasia, and neoplastic nodules and carcinomas (200 mg/kg groups only) of the liver were induced by MCB in rats. Liver tumor incidence data for combined experiments in rats were: males — 5% in controls, 0% in 100 mg/kg group, 14% in 200 mg/kg group, and 77% in 400 mg/kg group; females — 0% in controls, 0% in 100 mg/kg group, 12% in 200 mg/kg group, and 63% in 400 mg/kg group. MCB was not shown to be carcinogenic in mice.

Key words: Methyl carbamate — Hepatocarcinogenesis — Rats

Methyl carbamate (MCB, $H_2N\text{-}CO\text{-}O\text{-}CH_3$) was evaluated in short- and long-term carcinogenesis studies in Fischer 344 rats and B6C3F1 mice in part because the ethyl analogue (ethyl carbamate, urethane, $H_2N\text{-}CO\text{-}O\text{-}CH_2CH_3$) had been shown to cause liver and mammary tumors in mice; liver, neurogenic, and thyroid gland tumors in rats,[1,2] and because previous studies of MCB were not considered adequate for evaluation.[3]

In mutagenesis studies, MCB was negative in *Salmonella typhimurium*,[4,5] *Escherichia coli*,[6] *Bacillus subtilis*,[7] or mouse L5178Y/TK$^{+/-}$ lymphoma[8–10] assays. No unscheduled DNA synthesis was observed in male F344 rat primary liver cells treated with MCB *in vitro*.[4] Exposure of cultured Chinese hamster ovary cells to MCB in the presence or absence of liver S9 did not increase the frequency of chromosomal aberrations or sister chromatid exchanges (SCE).[4]

In *in vivo* studies, no significant increase in the number of sex-linked recessive lethal mutations was detected in *Drosophila melanogaster* injected with MCB.[4] Intraperitoneal administration of MCB to intact or hepatectomized mice did not induce SCE in alveolar macrophages and bone marrow cells or in regenerating liver cells.[11] In a dominant lethal mutation study no increase in the frequency of early fetal death or preimplantation losses was observed.[12] In mouse bone marrow micronucleus test MCB administered intraperitoneally did not increase the frequency of micronucleated polychromatic erythrocytes or the percentage of polychromatic erythrocytes.[13] MCB given intravenously to B6C3F1 mice did not cause alteration in immune functions.[14]

In carcinogenesis studies, MCB injected intraperitoneally[15,16] or subcutaneously[17] did not induce lung adenomas in strain A mice. Tumor incidences in mice given a single subcutaneous injection of MCB followed by weekly topical applications of croton oil were similar to those of the controls.[18,19] Mice given 15 weekly topical applications of MCB followed by applications of croton oil for 18 weeks did not have higher incidences of tumors compared with controls.[20]

We reported that MCB (400–500 mg/kg), when administered by gavage for 13 weeks to F344 rats, produced morphologic changes in the liver,[21] consistent with those that often precede the development of hepatocellular neoplasia.[22] The changes consisted of proliferation of hepatocytes characterized by basophilic and other foci of cellular alteration and, in addition, frequent mitoses with atypical forms, hepatocellular necrosis of a focal nature, and pigmentation of Kupffer's cells. Lesions were not seen in the liver or any other organ in B6C3F1 mice given MCB (1,000 mg/kg) in companion experiments. In the present paper we report that gavage administration of MCB induced hepatocellular adenomas and carcinomas in F344/N rats but not in B6C3F1 mice.

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MATERIALS AND METHODS

MCB was obtained from Millmaster Chemical Co., New York, NY. Analyses by infrared, ultraviolet, nuclear magnetic resonance spectroscopy and gas chromatography showed that the compound was at least 98% pure. The major impurity (1.2%) was identified as (N-methoxymethyl)methyl carbamate. Periodic analyses by infrared spectroscopy and gas chromatography indicated that no deterioration of the compound occurred over the course of the 2-year study and that an aqueous solution of MCB at room temperature was stable for at least 7 days. Fischer 344/N rats and B6C3F1 mice (6 weeks old) were obtained from Frederick Cancer Research Center, Frederick, MD and quarantined for 19 days before being placed on study. The animals were housed, 5 per cage, in polycarbonate cages in a room with controlled temperature (23°C), humidity (50%), and lighting (12 h). NIH 07 ration (Zeigler Bros., Gardners, PA) and tap water were available *ad libitum*. All animal husbandry operations were conducted under NIH guidelines.[23]

Two experiments with each species were conducted concurrently. In experiment 1, groups of 30 male and 30 female Fischer 344/N rats were administered MCB in distilled water by gavage at 0 or 400 mg/kg body weight and groups of 30 male and 30 female B6C3F1 mice were given MCB by the same route at 0 or 1,000 mg/kg body weight, 5 times a week. Ten animals from each group were killed and necropsied after 6, 12, and 18 months of MCB administration.

In experiment 2, groups of 50 male and 50 female F344/N rats were administered MCB at 0, 100, or 200 mg/kg and groups of 50 male and 50 female B6C3F1 mice were given the compound in water by gavage at 0, 500, or 1,000 mg/kg, 5 times/week for 103 weeks. The dose levels selected for experiments 1 and 2 were based on histopathology observations, body weights, and survival in 90-day studies.[21]

During the course of the study the animals were observed two times per day. Clinical signs were recorded and moribund animals were killed and necropsied. Body weights were recorded once per week for the first 12 weeks and every four weeks thereafter. At necropsy at the end of the experiments, multiple organs and tissues of each animal were examined for grossly visible lesions. Tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and evaluated histologically. All gross lesions as well as at least one tissue section of each organ were examined microscopically. More details are available in the NTP Technical Report.[4] Differences in survival were analyzed by life table methods.[24] Tumor incidence data were analyzed by survival-adjusted methods[25] and by Fisher's exact tests and Cochran-Armitage trend tests based on the overall proportion of tumor-bearing animals.[26] *P*-values reported for tumor comparisons are one-sided.

RESULTS

Rat studies

Experiment 1. The survival and body weights of rats administered MCB at 0 or 400 mg/kg for 6, 12, and 18

Table I. Survival and Mean Body Weights of Rats in the Six-, Twelve-, and Eighteen-Month Studies of Methyl Carbamate

Time	Dose (mg/kg)	Survival[a]	Mean body weights (g)	
			Initial	Final
Male				
6-month	0	10/10	180±3.6[b]	452±6.1
	400	10/10	173±4.2	372±6.2[c]
12-month	0	10/10	175±4.0	496±10.2
	400	9/10	173±3.8	432±11.4[c]
18-month	0	10/10	190±4.8	505±13.6
	400	1/10	169±13.0	—
Female				
6-month	0	10/10	127±2.7	223±4.2
	400	10/10	127±3.0	214±3.3
12-month	0	10/10	127±2.8	285±5.0
	400	10/10	128±3.0	257±7.3[c]
18-month	0	10/10	133±5.0	324±9.0
	400	8/10	128±3.5	260±7.4[c]

a) Number surviving/number initially in group.
b) Mean±standard error of the mean.
c) $P<0.01$ vs. control (Student's *t* test).

Table II. Adrenal Gland and Liver Weights of Rats in the Six-Month Studies of Methyl Carbamate

Organ	Dose (mg/kg)	Organ weight[a] (mg)	Organ/body weight ratio ($\times 10^{-3}$)
Male			
Liver	0	15,950±966[b]	35.3±0.91
	400	12,900±1,135[c]	34.7±2.40
Adrenal gland	0	52.8±3.3	0.117±0.005
	400	36.6±2.8[c]	0.099±0.008[c]
Female			
Liver	0	7,260±593	32.5±1.44
	400	6,480±540[c]	30.3±1.66[c]
Adrenal gland	0	55.9±3.0	0.251±0.011
	400	40.8±3.3[c]	0.191±0.015[c]

a) In each group organs from 9–10 animals were weighed.
b) Mean±standard deviation.
c) $P<0.01$ vs. control (Student's *t* test).

months are presented in Table I. Survivals of the vehicle control and MCB-treated rats at the 3 time periods were similar except for treated male rats at 18 months. This lower survival was due to the killing of moribund tumor-bearing animals prior to the scheduled necropsy day. MCB administration caused a reduction in body weight gains in the male and female rats after 3 months.

At 6 months, the adrenal gland and liver weights of the MCB exposed male and female rats were reduced (Table II). Microscopically, the adrenal glands of the treated male and female rats appeared to be normal. On gross examination, the livers of the MCB-treated male and female rats had numerous tan, yellow or white areas, occasionally accompanied by diffuse mottling of all liver lobes. Microscopically, cytologic changes and proliferative lesions involving multiple liver lobules were observed. These consisted of mixtures of hepatocytes with vacuolated or basophilic cytoplasm, atypical nuclei, and abnormal mitoses. Neoplastic nodules of the liver were observed in 6/10 dosed males and 5/10 dosed females (Table III). Histopathologic changes were not observed in any of the vehicle controls (Table III).

At 12 months, cytologic alteration of the hepatocytes similar to that occurring at 6 months was observed in all dosed male and female rats (Table III). Neoplastic nodules of the liver were observed in 7/10 dosed males and 9/10 dosed females. Hepatocellular carcinomas were observed in 8/10 dosed males and 6/10 dosed females.

At 18 months, neoplastic nodules of the liver were observed in 2/10 dosed males and 5/10 dosed females. Hepatocellular carcinomas were observed in 9/10 dosed males and 8/10 dosed females (Table III). The hepatocellular carcinomas in the male rats were moderately to poorly differentiated and consisted of multiple nodules of hepatocytes with prominent trabeculation and infiltration of adjacent parenchyma. Individual hepatocytes had distinct basophilic cytoplasm with large prominent vesicular nuclei and usually one large nucleolus. Mitoses were generally frequent. Metastases were seen in 7/10 of the male rats (5 to lungs, 1 to seminal vesicles, 1 to spleen). Metastasis did not occur in the female rats. In most instances exposed animals had multiple tumors. In the vehicle control male and female rats cytologic alterations but no neoplastic lesions were observed at 12 and 18 months.

Experiment 2. Survivals of male and female rats treated with 100 or 200 mg/kg at 24 months (males: 26/50, 29/50; females: 36/50, 34/50) were not statistically different from those of their respective controls (males: 19/50; females: 29/50). Mean body weights of male and female rats treated at 100 mg/kg were similar to those of their respective controls, while those of rats treated at 200 mg/kg were generally 5–9% lower.

Liver lesions observed in the male and female rats are presented in Table IV. Incidences of chronic focal inflammation, cytologic alteration, and hyperplasia, were significantly ($P<0.05$) higher in female rats treated at 200 mg/kg than in controls. The incidence of cytologic alteration was also significantly elevated in male rats receiving 200 mg/kg and in female rats receiving 100 mg/kg. Increased incidences of chronic focal inflammation and hyperplasia in male rats receiving 200 mg/kg were not statistically significant, due in part to the improved survival in this group relative to controls.

The incidence of liver neoplasms was significantly ($P<0.05$) elevated in female rats receiving 200 mg/kg (6/49) relative to controls (0/50; see Table IV). Four of these lesions were neoplastic nodules, and one was a hepatocellular carcinoma, and one animal had both a neoplastic nodule and a hepatocellular carcinoma. The

Table III. Incidence of Lesions of the Liver in Rats in the Six-, Twelve-, and Eighteen-Month Studies of Methyl Carbamate

Lesion	Time (months)	Male		Female	
		Control	400 mg/kg	Control	400 mg/kg
Cytologic alteration	6	0/10	10/10[a]	0/10	10/10[a]
	12	2/10	10/10[a]	3/10	10/10[a]
	18	7/10	8/10	9/10	10/10
Neoplastic nodule	6	0/10	6/10[a]	0/10	5/10[b]
	12	0/10	7/10[a]	0/10	9/10[a]
	18	0/10	2/10	0/10	5/10[b]
Hepatocellular carcinoma	6	0/10	0/10	0/10	0/10
	12	0/10	8/10[a]	0/10	6/10[a]
	18	0/10	9/10[a]	0/10	8/10[a]

a) $P<0.01$, vs. controls (Fisher's exact test).
b) $P<0.05$, vs. controls (Fisher's exact test).

Table IV. Number of Rats with Liver Lesions in the Tow-Year Studies of Methyl Carbamate

Lesion	Male			Female		
	0	100 mg/kg	200 mg/kg	0	100 mg/kg	200 mg/kg
No. examined	50	50	49	50	50	49
Chronic focal inflammation	2	3	9	13	17	31[a]
Cytologic alteration	14	11	30[b]	25	40[a]	46[a]
Hyperplasia	5	11	12	6	2	16[b]
Neoplastic nodule	3	0	3	0	0	5[b]
Hepatocellular carcinoma	1	0	4	0	0	2
Neoplastic nodules/carcinoma	4	0	7	0	0	6[b]

a) $P<0.01$, vs. controls (survival-adjusted analyses).
b) $P<0.05$, vs. controls (survival-adjusted analyses).

incidence of these tumors was marginally elevated, but not statistically significantly, in male rats receiving 200 mg/kg (7/49) compared with that in controls (4/50). No morphological difference was observed between the lesions found in the two groups of male rats.

Mouse studies

Experiment 1. All MCB control and treated male female mice were alive and appeared healthy at the 6-, 12-, and 18-month scheduled terminations. At each of these time intervals body weights of the treated male and female mice were about 10–15% lower than their respective controls. No compound-related histopathologic change was observed in the liver of male and female mice. However, the relative liver weights of male and female mice at 6 months were significantly ($P<0.05$) higher than those of the controls. Liver weights were not taken at the 12 and 18 month intervals.

Experiment 2. Survival rates between the vehicle control and MCB-exposed male (28/50, 35/50, 28/50) and female (38/50, 36/50, 31/50) mice were not different. Final body weights of male mice in the 500 and 1,000 mg/kg groups were 6% and 18% lower and those of female mice were 16% and 33% lower than those of the vehicle controls, respectively. Incidences of nonneoplastic and neoplastic lesions of the two exposed groups of male and female mice were not significantly elevated relative to their respective controls.

DISCUSSION

The present studies demonstrate that MCB causes liver neoplasia in both male and female rats, and these effects were seen as early as 6 months. At the 400 mg/kg MCB exposure level, cytologic alterations were induced in all male and female rats in 6, 12, and 18 months compared to controls (Table III). At each of these intervals, 50–90% of the MCB-exposed rats had either neoplastic nodules, hepatocellular carcinoma, or both. At one-half this level (200 mg/kg) for 2 years, MCB induced significant increases of hepatocellular neoplasms in female rats but only marginally in male rats. Unfortunately, at the 24-month time period no 400 mg/kg dose group was included, largely because there was some concern during the design of those experiments that well-being, growth, and survival would be compromised. In the groups receiving 100 mg/kg for 2 years MCB had no apparent influence on hepatocarcinogenesis in male and female rats; the only nonneoplastic effects caused by MCB administration were cytologic alterations in female rats.

These results show that MCB-induced carcinogenicity and earlier occurrence of tumors are dose-related. These findings are the first demonstration of a carcinogenic effect of MCB in rodents. The results are particularly significant from a scientific and public health point of view in that hitherto MCB has been referred to as a noncarcinogenic analogue of urethane.[11, 26, 27]

The mechanism of carcinogenic action of MCB in rats is not known.[28] MCB has not been shown to induce mutations in bacterial or mammalian cell systems, *in vitro* or *in vivo*. MCB did not bind to rat liver DNA,[4, 29] and only trace binding to mouse liver DNA has been detected,[4, 30, 31] although binding to mouse dermal and epidermal DNA was readily demonstrated.[19, 32] Unscheduled DNA synthesis was not observed in perfused liver cells of F344 male rats exposed to MCB *in vitro*.[4] Thus, the possibility of methyl carbamate forming DNA adducts or causing DNA damage leading to infidelity in DNA replication appears unlikely. As yet, however, no molecular examination of liver tumors has been done on proto-oncogene activation or suppressor gene inactivation. That MCB exerts carcinogenic action similar to that of peroxisome proliferators also appears unlikely

since the present study showed that MCB causes a reduction in rat liver weight whereas peroxisome proliferators generally elevate liver weight.[33] On the other hand, MCB may cause changes in the methylation patterns or the tertiary structure of DNA, as has been proposed for some chemical carcinogens of unknown genotoxicity or considered to be nongenotoxic.[34]

As far as is known, the induction of hepatocarcinogenesis by genotoxic chemicals follows a common sequence: altered cellular foci appear first, followed by clonal expansion to neoplastic nodules, and then hepatocellular carcinomas.[22, 35–38] Although MCB is "nongenotoxic" the sequence of cellular changes in rat liver as shown in experiment 1 appears to be quite similar to that induced by known genotoxic hepatocarcinogens. Indeed, proliferative liver lesions were detected within 90 days of MCB administration.[21] Recently, the expression of glutathione *S*-transferase placental form (GST-P) in altered hyperplastic foci has been studied extensively as a biomarker in rat hepatocarcinogenesis.[39] However, GST-P expression in the MCB-induced foci has not been reported.

The present studies also demonstrated that methyl carbamate at doses up to 1,000 mg/kg for 2 years was not carcinogenic in mice, and confirmed results obtained by other investigators.[15–20] These previous reports were considered by IARC[3] as being inadequate for the purpose of evaluating the carcinogenic potential of MCB due to relatively short durations of MCB exposure and observation. The present studies demonstrate that MCB did not induce any apparent carcinogenic response in mice at daily exposures of 500 or 1,000 mg/kg for 2 years.

The rates of MCB metabolism in rats and mice may play an important role in determining the toxicity and carcinogenicity of MCB in these two species. Ioannou *et al.*[30] and Ioannou and Matthews[40] reported that absorption and tissue distribution of MCB were similar in both species and were apparently independent of dose in a range of 0.4–100 mg/kg in both species. The products of MCB metabolism (carbon dioxide in exhaled air and parent compound in urine) were the same in both species and only the parent compound was detected in tissues of either species. However, the rates of metabolism and elimination of MCB as carbon dioxide were slower in rats than in mice. The half-life of MCB in rats was approximately 3 days compared to approximately 16 hours in mice. Boyland and Papadopoulos[41] previously reported that intraperitoneally injected MCB was retained in rat liver and various other tissues for up to 5 days. The slow elimination of MCB in rats probably resulted in higher accumulation of the compound in liver and may account for the toxic and carcinogenic effects induced in liver. Whether this helps to explain the tumor response differences remains to be investigated.

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