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Rapid Communication

Carcinogenicity and Chronic Toxicity in Rats and Mice Exposed to Chloroform by Inhalation

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Abstract: Carcinogenicity and Chronic Toxicity in Rats and Mice Exposed to Chloroform by Inhalation: Seigo YAMAMOTO, *et al.* Japan Bioassay Research Center—A bioassay study of carcinogenicity and chronic toxicity of chloroform was undertaken by inhalation exposures of groups of 50 F344 rats and 50 BDF_1 mice of both sexes to chloroform for 6 h/d × 5 d/wk × 104 wk. The exposure concentration was 0 (control), 10, 30 or 90 ppm for rats and 0, 5, 30 or 90 ppm for mice. Combined incidences of renal cell adenomas and carcinomas, and of hepatocellular adenomas and carcinomas increased in the exposed male and female mice, respectively. Incidences of atypical tubule hyperplasia, cytoplasmic basophilia and nuclear enlargement in the kidneys and fatty change in the liver increased in the exposed male mice. Increased incidence of altered cell foci in the exposed female mice was causally related to the hepatocellular adenomas and carcinomas. No significantly increased incidence of the kidney or liver tumors was observed in the exposed rats of either sex. Increased incidences of nuclear enlargement and dilatation of tubular lumen were found in the kidneys of exposed rats. No-observed-adverse-effect-levels (NOAELs) for the biologically significant endpoint were determined from the dose-response relationships of the present datasets. The NOAEL for the histopathological endpoint of the kidneys resulted in 5 ppm for mice and 10 ppm for rats. An occupational exposure limit for chloroform was discussed in light of the NOAELs.
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Key words: Tumor, Chloroform, Rat, Mouse, NOAEL, Liver, Kidney, Occupational Exposure Limit

Chloroform is known as an ubiquitous environmental contaminant found in multiple media including community drinking water, outdoor and indoor air and some foodstuffs[1, 2]. Chloroform has been widely used as an organic solvent, an intermediate for the synthesis of fluorocarbons and drugs and an insecticidal fumigant. Chloroform poses a potential hazard to the health of community residents and workers exposed to it. Chloroform is classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC)[3] and the Japan Society for Occupational Health (JSOH)[4], and as a confirmed animal carcinogen with unknown relevance to humans (A3) by the American Conference of Governmental Industrial Hygienists (ACGIH)[5]. The majority of studies show that chloroform is without measurable genotoxic activity[6, 7]. The carcinogenicity of chloroform has been evaluated by bioassay studies of rodents on long-term oral administration of chloroform in corn oil by oral gavage[8], in toothpaste by gavage[9] and in water by drinking[10]. Nevertheless, no bioassay study of carcinogenicity and chronic toxicity in long-term inhalation exposures of rodents to chloroform vapor has been reported so far. Such data obtained from the inhalation exposures of rodents[11–14] would be more important for health risk assessment of workers and community residents exposed to chloroform than those obtained by other routes of exposure, because inhalation exposure is a principal route for exposure of workers and community residents to volatile chloroform[15].

The present study was intended to examine the carcinogenicity and chronic toxicity of chloroform by exposing F344 rats and BDF_1 mice of both sexes to chloroform vapor at different concentrations by inhalation for 104 wk. The results reported here were focused on neoplastic and non-neoplastic lesions of the kidneys and liver. No-observed-adverse-effect-levels (NOAELs) for the biologically significant endpoint were determined from dose-response relations for the non-neoplastic and neoplastic lesions in the chloroform-exposed animals. An occupational exposure limit for chloroform was discussed in light of the NOAELs.

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Materials and Methods

Chemicals

Chloroform of reagent grade (greater than 99% in purity) was obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). The chemical was received as a liquid in 500 g bottles and was stored at room temperature in a testing laboratory room throughout the study period. Each lot of chloroform used in the present study was analyzed for its stability and purity by gas chromatography and infrared spectrometry at the termination of its use, and no decomposition product or impurity was observed.

Animals

F344/DuCrj rats and Crj:BDF_1 mice of both sexes were obtained at the age of 4 wk from Charles River Japan, Inc (Kanagawa, Japan). The animals were quarantined and acclimated for 2 wk before the start of the experiment, and were housed individually in stainless-steel wire hanging cages in stainless steel inhalation exposure chambers maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 10% with 12 air changes per hour. The four exposure chambers were installed in a barrier system animal room maintained at the above temperature and humidity with 15–17 air changes per hour. Fluorescent lighting was controlled automatically to give a 12-h light/dark cycle. All rats and mice had free access to a commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water.

Exposure to chloroform

A chloroform vapor-air mixture was generated by bubbling clean air through the liquid chloroform, further diluted with clean air, and supplied to the inhalation exposure chambers by the method and apparatus described previously[16]. The concentration of chloroform vapor in the exposure chambers was maintained at 5.0 ± 0.1 (mean ± SD), 30.0 ± 0.1 or 90.1 ± 0.8 ppm for mice and 10.1 ± 0.1, 30.0 ± 0.4 or 89.9 ± 0.7 ppm for rats throughout the exposure period.

Experimental design

Groups of 50 male and 50 female rats were exposed to airflow containing chloroform vapor at a target concentration of 10, 30 or 90 ppm for 6 h (10:00–16:00) /day × 5 d/wk × 104 wk. Groups of 50 male and 50 female mice were exposed to airflow containing chloroform at a target concentration of 5, 30 or 90 ppm for 6 h/d × 5 d/wk × 104 wk. Because both 30 ppm- and 90 ppm-exposed mice were found to die of acute poisoning in the first week of exposure in the preliminary experiment, the exposure concentrations for both 30 and 90 ppm groups of mice were increased stepwise as follows[17]. For the 30 ppm group, the mice were exposed to 5 ppm for the first 2 wk, 10 ppm for another 2 wk and 30 ppm for the subsequent 100 wk. For the 90 ppm group, the mice were exposed to 5 ppm for the first 2 wk, 10 ppm for the following 2 wk, 30 ppm for the next 2 wk and 90 ppm for the subsequent 98 wk. Groups of 50 rats and 50 mice of both sexes, serving as controls, were handled in the same manner as the chloroform-exposed groups, but were exposed to clean air.

Clinical observations and analysis, and pathological examinations

The animals were observed daily for their clinical signs and mortality. They were weighed and their food, and water consumptions were measured weekly for the first 13 wk of the study period and every 4 wk thereafter. Animals found dead, in a moribund state or surviving to the end of the 2-yr exposure received complete necropsy. Urinary, hematological and blood biochemical parameters of all surviving animals were measured obtained from urine collected at the end of the 2-yr exposure period and from blood samples taken under etherization at the end of the 2-yr exposure period after overnight fasting. All organs were removed, weighed at necropsy and examined for macroscopically visible lesions. The urinary, hematological and blood biochemical parameters examined here were given in the OECD guidelines[18], and the histopathologically examined tissues were described in detail in our previous paper[19]. The tissues for microscopic examination were fixed in 10% neutral buffered formalin, embedded in paraffin, and sections of all tissues and tumors were 5μm thick, and stained with hematoxylin and eosin.

Statistical analysis

The incidence of non-neoplastic lesions and urinary data were analyzed by chi-square test. The incidence of neoplastic lesions was statistically analyzed by Peto's test[20] and Fisher's exact test. Body weight, food consumption, and hematological and blood biochemical parameters were analyzed by Dunnett Test. The method for application of the statistical tests to those data was given in the previous paper[19].

Results

Mice Study

Survival, body weight, clinical signs and food consumption

There was no difference in the 2-yr survival rate between the exposed groups of both sexes and the respective control groups (for males, control group: 33/50, 5 ppm group: 38/50, 30 ppm group: 36/50, 90 ppm group: 35/48, and for females, control group: 29/50, 5 ppm group: 36/49, 30 ppm group: 25/50, 90 ppm group: 24/48). In males, body weights of the three exposed groups significantly decreased over the control group throughout the study period. Body weight of all

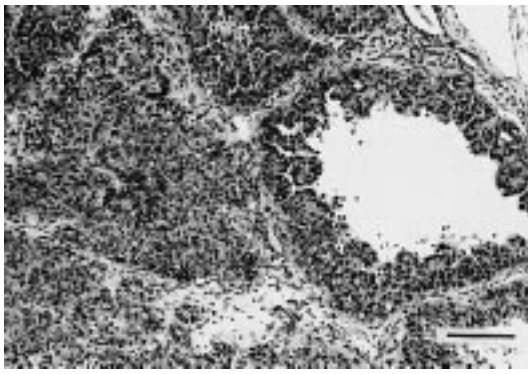

Fig. 1. Renal cell carcinoma in a male mouse exposed to 90 ppm chloroform by inhalation for 104 wk. H.E. stain. Bar indicates 100 μm.

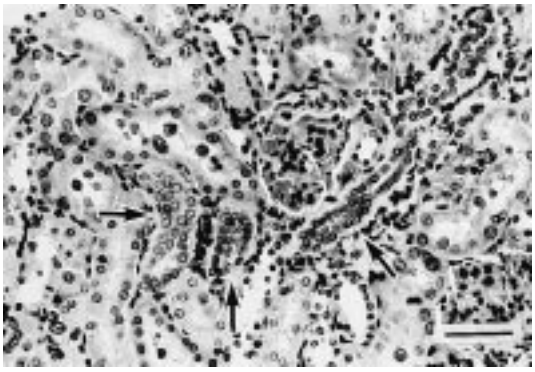

Fig. 2. Cytoplasmic basophilia (arrow) in a kidney of a male mouse exposed to 90 ppm chloroform by inhalation for 104 wk. H.E. stain. Bar indicates 50 μm.

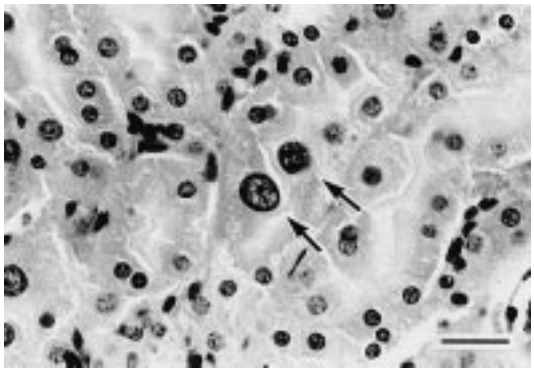

Fig. 3. Nuclear enlargement (arrow) in a proximal tubule of a kidney in a male mouse exposed to 90 ppm chloroform for 104 wk. H.E. stain. Bar indicates 25 μm.

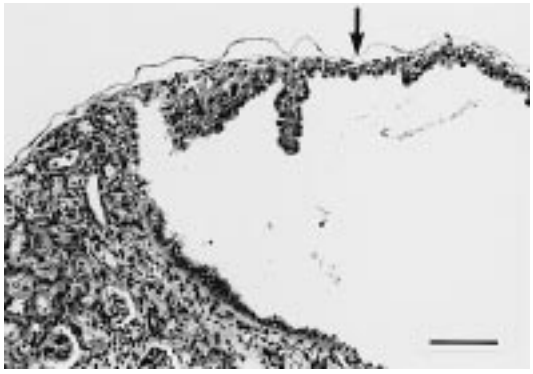

Fig 4. Atypical tubule hyperplasia (arrow) in a male mouse exposed to 90 ppm chloroform by inhalation for 104 wk. The renal tubule shows the cystic dilatation and the papillary projection, but almost all the tubular epithelium is single-layered as yet. H.E. stain. Bar indicates 100 μm.

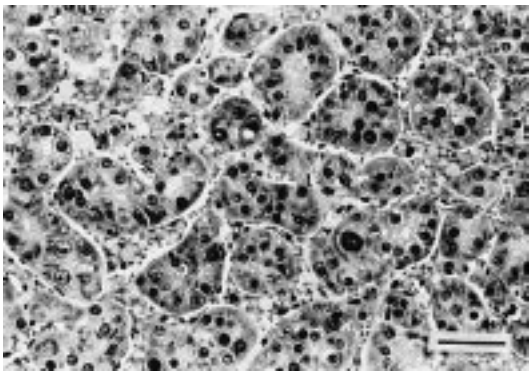

Fig. 5. Hepatocellular carcinoma in a female mouse exposed to 90 ppm chloroform by inhalation for 104 wk. H.E. stain. Bar indicates 50 μm.

chloroform-exposed female groups significantly decreased over the control, but the significant decrease in the body weight of the 5 ppm- and 30 ppm-exposed females was recovered to the control levels in the 52nd and 94th week of the repeated exposure, respectively. No overt clinical signs were observed in either the control or exposed groups. Food consumption of the chloroform-exposed mice of both sexes was not statistically different from that of the respective controls throughout the study period.

Macroscopic findings and organ weight

The incidence of renal nodules significantly increased in the 30 and 90 ppm-exposed male group, as compared to the control (data not shown). The absolute kidney weight of the 90 ppm-exposed male group significantly increased over that of the control, but the relative kidney weight did not reach statistical difference because of a large standard deviation due to the occurrence of the kidney tumors (data not shown).

Neoplastic and non-neoplastic lesions in the kidneys and liver

Kidney: The incidence of renal cell carcinomas (Fig. 1) in the exposed males increased in a dose-related

manner as indicated by a significant positive trend by Peto's test. Kidney tumors less than 3 mm in size in the histological sections without marked atypia were diagnosed as renal cell adenoma. When the cases of renal cell carcinomas were combined with those of the renal cell adenomas, the combined incidence also gave a significant positive trend by Peto's test, and significantly increased in the 30 ppm- and 90 ppm-exposed males (Table 1). No kidney tumor occurred in either control or exposed female groups. The exposed males exhibited significantly increased incidences of cytoplasmic basophilia (Fig. 2), nuclear enlargement (Fig. 3) and atypical tubule hyperplasia (Fig. 4) at 30 ppm and 90 ppm. The 90 ppm-exposed females exhibited a significantly increased incidence of the cytoplasmic basophilia (Table 2).

Liver: The incidence of hepatocellular carcinomas (Fig. 5) occurring in the exposed female groups (1/49, 0/50, 3/48) exhibited a significant positive trend by Peto's test ($P<0.02$) (Table 1). Although the difference in the incidence of hepatocellular adenomas was not statistically significant between the exposed female groups and the control, the combined incidence of hepatocellular adenomas and carcinomas exhibited a significant positive trend by Peto's test. Furthermore, a significantly increased incidence of total altered cell foci was found in the 90 ppm females (Table2). A significant positive trend by Peto's test was observed in the incidence of hepatocellular carcinomas and the combined incidence of hepatocellular adenomas and carcinomas for the exposed male groups, but there was no statistical difference in those tumor incidences between the 90 ppm male group and the control, because many cases of these two kinds of liver tumors were observed in the control males. The incidences of these liver tumors in the 90 ppm-exposed males were approximately the same as the

Table 1. Incidences of neoplastic lesions in the mice and rats exposed to chloroform vapor at different concentrations for 104 wk

(A) Mice

	Male					Female				
Group	Control	5 ppm	30 ppm	90 ppm	Peto	Control	5 ppm	30 ppm	90 ppm	Peto
Number of animals examined	50	50	50	48		50	49	50	48	
Liver										
Hepatocellular adenoma	5	7	6	8		1	1	4	3	
Hepatocellular carcinoma	10	0**	7	10	↑	1	1	0	3	↑
Hepatocellular adenoma + carcinoma	14	7	12	17	↑	2	2	4	6	↑↑
Hemangioma	0	0	1	0		0	0	0	0	
Hemangiosarcoma	3	0	2	1		2	0	0	1	
Histiocytic sarcoma	2	0	0	0		0	0	1	0	
Kidneys										
Renal cell adenoma	0	0	3	1		0	0	0	0	
Renal cell carcinoma	0	1	4	11**	↑↑	0	0	0	0	
Renal cell adenoma + carcinoma	0	1	7*	12**	↑↑	0	0	0	0	

(B) Rats

	Male					Female				
Group	Control	10 ppm	30 ppm	90 ppm	Peto	Control	10 ppm	30 ppm	90 ppm	Peto
Number of animals examined	50	50	50	50		50	50	50	49	
Liver										
Hepatocellular adenoma	0	0	0	0		1	0	2	1	
Kidneys										
Renal cell adenoma	0	0	0	0		0	0	0	1	
Pituitary gland										
Adenoma	22	23	21	17		24	20	18	11*	

*: $P \leq 0.05$, **: $P \leq 0.01$ Fisher Exact Test, ↑: $P \leq 0.05$, ↑↑: $P \leq 0.01$ Peto's Test

Table 2. Incidences of selected non-neoplastic lesions in the liver and kidneys of mice and rats exposed to chloroform vapor for 104 wk

(A) Mice

Group	Male				Female			
	Control	5 ppm	30 ppm	90 ppm	Control	5 ppm	30 ppm	90 ppm
Number of animals examined	50	50	50	48	50	49	50	48
Liver								
Necrosis: central	0	0	0	3	1	0	1	2
Necrosis: focal	1	2	6	2	0	0	2	3
Fatty change	4	2	6	24**	0	0	0	6*
Total altered cell foci	10	1**	1**	5	0	1	2	6*
Clear cell foci	6	0*	0*	3	0	1	0	3
Basophilic cell foci	3	1	1	1	0	0	1	2
Mixed cell foci	1	0	0	1	0	0	1	1
Kidneys								
Nuclear enlargement : proximal tubules	0	3	43**	42**	0	0	0	4
Cytoplasmic basophilia[a] +	33	40	8**	9**	0	4	3	5*
2+	7	1	36	34	0	0	0	2
3+	0	0	2	0	0	0	0	0
Atypical tubule hyperplasia	0	0	11**	14**	0	0	0	0
Tubular necrosis: proximal tubules	0	0	1	2	1	0	0	0

(B) Rats

Group	Male				Female			
	Control	10 ppm	30 ppm	90 ppm	Control	10 ppm	30 ppm	90 ppm
Number of animals examined	50	50	50	50	50	50	50	49
Liver								
Total altered cell foci	11	16	16	18	15	9	20	26
Clear cell foci	4	4	5	6	4	1	2	7
Acidophilic cell foci	2	5	2	3	0	1	0	1
Basophilic cell foci	4	6	8	8	7	5	10	4
Mixed cell foci	1	1	1	1	4	2	6	9
Vacuolated cell foci	0	0	0	0	0	0	2	5*
Kidneys								
Nuclear enlargement : proximal tubules	0	0	5*	32**	0	0	6*	34**
Dilatation : tubular lumen	0	0	9*	27**	0	0	5*	38**
Chronic progressive nephropathy[b] +	3	11*	10**	17**	8	19**	27**	15**
2+	6	10	24	14	15	7	5	3
3+	19	15	8	2	14	3	3	1
4+	19	8	2	1	4	2	0	2

Significant difference at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) by Chi square test. a) The severity of cytoplasmic basophilia was qualitatively scored as follows: +, a few lesions involving a single tubule in the whole histological section; 2+, more than 4 lesions involving two or more tubules in the whole histological section; 3+, numerous lesions throughout whole section. b) The severity of chronic progressive nephropathy was classified into four different grades according to the criteria described by Kawai[21].

average values in the historical control data in the Japan Bioassay Research Center. Other liver tumors including hemangioma, hemangiosarcoma and histiocytic sarcoma occurred in the exposed and control groups of male and female mice, but there was no significant difference in the tumor incidences between the exposed groups and the respective controls (Table 1).

Statistically significant, exposure-related increases in the incidences of fatty change in the 90 ppm-exposed males and females and of total altered cell foci in the 90 ppm-exposed females were noted. The incidence of central and focal necrosis of the liver in both males and females tended to increase in an exposure-related manner, although the difference did not reach statistical

significance (Table 2).

Other organs: There was no remarkable change in the occurrence of neoplastic or non-neoplastic lesions in the other organs except for the nasal cavity. Thickening of the bone at 5 ppm and above for both sexes, atrophy and respiratory metaplasia of the olfactory epithelium in the 90 ppm males and in the 5 ppm and above-exposed females were noted.

Urinary, hematological and biochemical parameters: The exposed males showed significantly increased serum

Table 3. Serum levels of blood biochemical parameters and urinalysis in mice and rats exposed to chloroform vapor for 104 wk

(A) Mice

Group	Male				Female			
	Control	5 ppm	30 ppm	90 ppm	Control	5 ppm	30 ppm	90 ppm
Number of animals examined	33	38	36	35	28	34	24	24
BIOCHEMISTRY								
Total protein (g/d*l*)	5.6	5.8	5.9	6.1**	5.5	5.5	5.6	6.0
Glucose (mg/d*l*)	175	185	166	166	136	129	140	156
Total cholesterol (mg/d*l*)	111	107	125	124	78	71	86	77
Triglyceride (mg/d*l*)	83	82	101	80	82	72	81	58**
BUN (mg/d*l*)	25.8	22.5	26.3*	30.8**	17.2	21.4	19.3	21.4**
GOT (IU/*l*)	85	58	115	111*	144	148	125	175*
GPT (IU/*l*)	29	16	40	44**	28	47	39	50**
LDH (IU/*l*)	316	263	388	390	745	560	1437	748
ALP (IU/*l*)	171	184*	219**	205**	264	322	235	303
URINALYSIS								
Glucose	0/33	0/39	0/38	0/35	0/29	0/36	1/27	1/24
Occult blood	5/33	2/39	5/38	1/35	5/29	2/36	1/27	2/24

(B) Rats

Group	Male				Female			
	Control	10 ppm	30 ppm	90 ppm	Control	10 ppm	30 ppm	90 ppm
Number of animals examined	27	39	36	38	37	35	40	34
BIOCHEMISTRY								
Total protein (g/d*l*)	6.7	7.1	7.0	6.9	7.0	7.4*	7.3	7.2
Glucose (mg/d*l*)	162	169	165	154	170	168	164	160
Total cholesterol (mg/d*l*)	173	164	153	125**	142	131	135	149
Triglyceride (mg/d*l*)	222	167	146*	87**	191	126	109	94**
Phospholipid (mg/d*l*)	289	268	241*	196**	280	252	255	271
Creatinine (mg/d*l*)	0.9	0.6	0.6**	0.7**	0.5	0.5	0.5	0.5
BUN (mg/d*l*)	28.6	20.6**	18.3**	23.2**	17.9	18.5	18.5	18.6
GOT (IU/*l*)	67	79	81	98*	128	113	124	144
GPT (IU/*l*)	21	25*	24	26*	37	40	39	48
γ-GTP (IU/*l*)	4	8*	10**	7*	4	4	5	6**
LDH (IU/*l*)	164	239	179	311	297	245	239*	344
ALP (IU/*l*)	215	265	283	243	152	133	157	174
URINALYSIS								
Glucose	0/27	0/39	2/37	20/39**	2/41	8/37*	29/41**	24/34**
Occult blood	3/27	6/39	4/37	10/39	2/41	1/37	4/41	2/34

Values shown are the means for each group, and the urinalysis data indicate the number of animals having positive glucose or occult blood/total number of animals examined.

Significant difference at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) by Dunnett's test for biochemistry and by chi-square for urinalysis.

BUN: blood urea nitrogen, GOT: glutamate oxaloacetate transaminase, GPT: glutamate pyruvate transaminase, ALP: alkaline phosphatase, γ-GTP: γ-glutamyl transpeptidase, LDH: Lactate dehydrdogenase, ALP: alkaline phosphatase

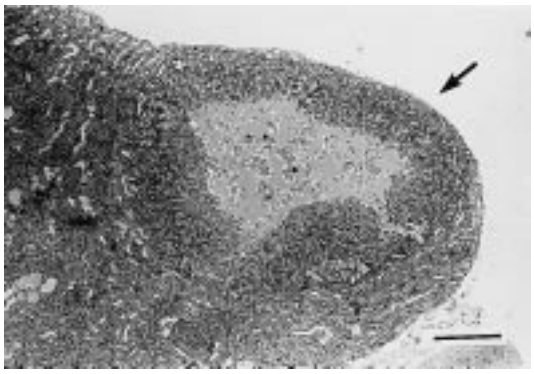

Fig. 6. Renal cell adenoma (arrow) in a female rat exposed to 90 ppm chloroform by inhalation for 104 wk. H.E. stain. Bar indicates 250 μm.

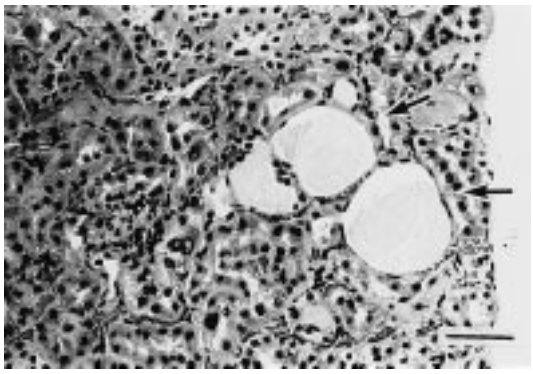

Fig. 7. Dilatation of tubular lumen (arrow) in a kidney of a female rat exposed to 90 ppm chloroform by inhalation for 104 wk. H.E. stain. Bar indicates 50 μm.

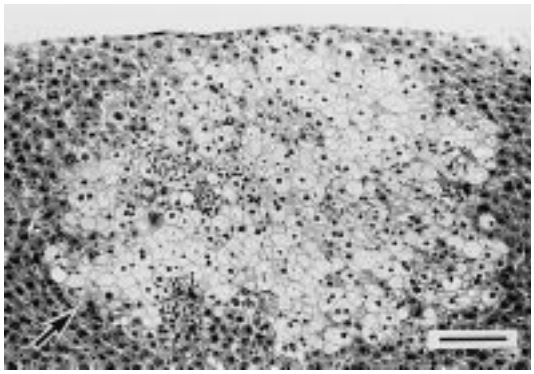

Fig. 8. Vacuolated cell focus (arrow) in the liver of a female rat exposed to 90 ppm chloroform by inhalation for 104 wk. H.E. stain. Bar indicates 100 μm.

levels of ALP at 5 ppm and above, BUN at 30 ppm and above, GOT and GPT at 90 ppm and total protein at 90 ppm. Significantly increased levels of triglyceride, BUN, GOT and GPT were observed in the 90 ppm-exposed females (Table 3). No significant changes in the hematological parameters were observed in the exposed mice.

Rats Study

Survival, body weight, clinical signs and food consumption

There was no statistical difference in the 2-yr survival rate between the exposed female groups and the respective control, but the survival rate of the control male group significantly decreased as compared to the exposed male groups (for males, control group: 27/50, 10 ppm group: 39/50, 30 ppm group: 37/50, 90 ppm group: 38/50, and for females, control group: 38/50, 10 ppm group: 36/50, 30 ppm group: 40/50, 90 ppm group: 34/49). The growth rates of the 90 ppm group of both sexes were significantly suppressed over the respective controls throughout the exposure period, but there was no statistical difference in body weight between the 10 and 30 ppm groups of both sexes and the respective controls. No overt clinical signs were observed among any control or exposed groups. Food consumption of the chloroform-exposed male and female groups was not statistically different from that of the respective controls throughout the study period.

Macroscopic findings and organ weight

No macroscopic lesion attributable to chloroform toxicity was seen in either males or females. Absolute and relative kidney weights of the 90 ppm-exposed female group were statistically higher than those of the control.

Neoplastic and non-neoplastic lesions in the kidneys and liver

Kidney: No statistically significant increase in the incidence of kidney tumors was observed in the exposed males and females, but a case of renal cell adenoma (Fig. 6) which occurred in a 90 ppm-exposed female (Table 1) was very rare, because the spontaneous occurrence of renal cell adenoma was 1/1048 for female F344 rats in the historical control data in the Japan Bioassay Research Center. Dose-related increases in the occurrence of nuclear enlargement of the proximal tubule and dilatation of the tubular lumen (Fig. 7) in the kidney were observed in the 30 ppm- and 90 ppm-exposed groups of both sexes. The severity of chronic progressive nephropathy (CPN) in the exposed males and females significantly decreased with an increase in the exposure concentration (Table 2).

Liver: Hepatocellular adenomas were observed in the female rats, but the incidence was not related to the exposure to chloroform (Table 1). There was no exposure-related, non-neoplastic liver lesion except for a significantly increased incidence of vacuolated cell foci

(Fig. 8) in the 90 ppm-exposed females (Table 2).

Other organs: There was no remarkable change in the occurrence of neoplastic or non-neoplastic lesions in the other organs except in the following three organs: The nasal lesions included thickening of the bone and atrophy and respiratory metaplasia of the olfactory epithelium at 5 ppm and above for both sexes. Lowered incidences of pituitary gland adenoma in the 90 ppm females and myocardial fibrosis in the 90 ppm males were noted.

Urinary, hematological and biochemical parameters: The exposed males exhibited significantly decreased serum levels of triglyceride, phospholipids and creatinine at 30 ppm and above, and total cholesterol at 90 ppm (Table 3). Serum levels of BUN significantly decreased in the three exposed groups of males, although those levels in the exposed females did not differ from the respective controls. GOT, GPT and γ-GTP significantly increased in the exposed males. The exposed females exhibited a significant decrease in triglyceride and an increase in γ-GTP at 90 ppm. Positive urinary glucose was observed in the 90 ppm-exposed males and in the 10 ppm and above-exposed females. No significant changes in the hematological parameters were observed in the exposed rats.

Discussion

So far, bioassay rodent studies of carcinogenicity and chronic toxicity of chloroform have been carried out only by oral administration of chloroform in corn oil or toothpaste by gavage or in water drinking. To the best of our knowledge, this is the first report demonstrating that 2-yr inhalation exposure of rats and mice of both sexes to chloroform vapor significantly increased the tumor incidence in mice but not in rats. The exposure concentrations of the 30 ppm- and 90 ppm-exposed groups of mice employed in the present study were increased stepwise during the first several weeks to the final target concentrations. The reason for using the stepwisely increased exposure concentrations is that acute deaths of male mice exposed to 30 ppm and 90 ppm can be prevented by challenging exposure to lower concentrations for the first several weeks[17]. The present results of no overt differences in the 2-yr survival rate, clinical signs and food consumption between the respective controls and the 30 ppm- and 90 ppm-exposed mice of both sexes can be taken to indicate that the stepwisely increased concentrations of 30 ppm and 90 ppm would not exceed the criteria for the maximum tolerable dose for long-term bioassay rodent studies on carcinogenicity[23].

Neoplastic lesions

Mice: The 2-yr inhalation exposure of male and female BDF_1 mice to chloroform vapor was found to significantly increase the combined incidence of renal cell adenoma and carcinoma in both 30 ppm- and 90 ppm-exposed males. The exposure-related occurrence of the renal cell tumors in male mice was supported by the additional evidence that the significantly increased incidence of atypical tubule hyperplasia was observed in the kidneys of both 30 and 90 ppm-exposed male mice. This atypical tubule hyperplasia can be classified as a pre-neoplastic lesion because of the histopathological similarity between atypical tubule hyperplasia and renal cell tumors. A combined incidence of hepatocellular adenoma and carcinoma in female mice significantly increased in a dose-related manner as indicated by Peto's test. As to liver cancer in female mice, Reuber[8] reported that oral administration of chloroform in corn oil by gavage significantly increased the incidence of hepatocellular carcinoma in $B6C3F_1$ mice administered 238 and 477 mg/kg/d. Jorgenson *et al*[10] reported that oral administration of chloroform in drinking water failed to increase the incidences of hepatocellular adenoma and carcinoma in the $B6C3F_1$ mice administered 263 mg/kg/d. In the present study, daily uptake of chloroform through the 6-h inhalation exposure of female BDF_1 mice to 90 ppm chloroform can be estimated as 105 mg/kg/d, assuming 24.5 m*l*/min as minute volume[24] and 90 % as the lung absorption ratio for chloroform[25]. Comparison of the above-mentioned daily uptake values for chloroform indicates that susceptibility to liver carcinogenicity in female mice depends on the strain of mice (BDF_1 vs $B6C3F_1$) and the route (inhalation vs ingestion) and mode (drinking vs gavage) of chloroform administration. In this context, Templin *et al.*[13] suggested a difference in the genetic background of hepatocarcinogen sensitivity genes (*Hcs*) between $B6C3F_1$ and BDF_1 mice as a causative factor in susceptibility to hepatic tumors. It is also noteworthy that approximately the same dose of chloroform taken by oral gavage induced hepatic tumors in female $B6C3F_1$ mice[8], whereas drinking of chloroform-containing water failed to induce hepatocellular carcinoma in the same strain of mice[10]. This difference is thought to be attributable to the bolus dose taken by oral gavage overwhelming hepatic defense mechanisms such as glutathione binding[26].

Rats: It was found in the present study that the 2-yr inhalation exposure of male and female F344 rats to 10, 30 or 90 ppm chloroform vapor does not result in any statistically significant, exposure-related increase in the incidences of liver and kidney tumors. So far, two long-term bioassay studies on the rat carcinogenicity of ingested chloroform have been reported: Reuber[8] reported that oral administration of chloroform in corn oil by gavage to the Osborne-Mendel rat significantly increased the incidences of hepatic cholangiofibroma and cholangiocarcinoma in the high-dosed females (200 mg/kg/d), and of kidney carcinoma in the high-dosed males (180 mg/kg/d), and of thyroid adenoma and carcinoma

in the low-dosed (100 mg/kg/d) and high-dosed females. Jorgenson *et al.*[10] reported that oral administration of chloroform in drinking water (160 mg/kg/d) significantly increased the incidence of renal tubular cell adenoma and adenocarcinoma in male Osborne-Mendel rats. An apparent difference between no induction of renal tumors in male F344 rats observed in the present study and significant induction of renal tumors in male Osborne-Mendel rats reported by Reuber[8] and Jorgenson *et al.*[10] might be attributed to a possible difference in the amount of chloroform dosed daily, in addition to a strain difference (F344 vs Osborne-Mendel rat). Assuming the minute volume as 215ml/min[24] and the lung absorption ratio of chloroform as 90%[25], the daily uptake of chloroform by the 90 ppm-exposed male F344 rats can be estimated as 70.7 mg/kg/d. It may not be irrational to infer that a daily dose of chloroform greater than 100 mg/kg resulted in the induction of renal tumors in rats.

As to the other neoplastic lesions, a lowered incidence of the pituitary gland adenomas, which is known to decrease with a decrease in body weight[27], was observed in the 90 ppm female rats (Table 1).

Non-neoplastic lesions

Mice: The significantly increased incidence of renal cytoplasmic basophilia, which was observed in both 30 and 90 ppm-exposed male mice and in the 90 ppm-exposed female mice, is indicative of the regenerative, proliferative response to the necrosis of proximal tubules. A dose-related increase in the incidence of nuclear enlargement occurs only in the male mice. The significantly but slightly increased serum levels of BUN in the exposed male and female mice also indicate a functional disorder of the kidneys. These non-neoplastic lesions as indicated by the histopathological and biochemical parameters are more profound in male than in female mice. We reported that the 2 and 13 wk inhalation exposures to chloroform vapor induced cytoplasmic basophilia in the proximal tubules in male mice[22]. Therefore, the present and previous results reveal that the cytoplasmic basophilia in the proximal tubules is sustained throughout the 2-yr period of inhalation exposure, and that the sustained cytoplasmic basophilia is in significant association with both the pre-neoplastic atypical tubule hyperplasia and the renal cell adenoma and carcinoma. Our results are consistent with the findings by Larson *et al.*[11] and Templin *et al.*[12]. Larson *et al.*[11] showed dose-related increases in histopathological lesions and a cell proliferation index as measured by incorporation of bromodeoxyuridine in the kidneys of $B6C3F_1$ mice exposed to chloroform at 30 ppm and 90 ppm for 13 wk. Templin *et al.*[12] demonstrated both replacement of the proximal tubule epithelium by regenerating cells characterized by cytoplasmic basophilia and increased cell proliferation of the kidney cortex in male but not in female BDF_1 mice exposed to 30 ppm and 90 ppm chloroform vapor for 13 wk. It can be inferred that the sustained cytoplasmic basophilia plays a crucial role in development of the chloroform-induced renal tumors through the pre-neoplastic atypical tubule hyperplasia. Taking into account the weight-of-evidence that neither chloroform nor its metabolites readily bind to DNA, and that chloroform does not produce carcinogenic effects primarily by a specific mutagenic mode of action[6, 7], the present findings would provide additional evidence to support the hypothesis that tumor development results secondarily from chemically induced cytolethality, release of nucleases into the nucleus, inflammation and regenerative cell proliferation caused by the toxicant in a non-genotoxic-cytotoxic-proliferative mode of action[7, 28, 29].

Mild changes in the necrosis and fatty change in the liver of the exposed male mice observed in the present study are consistent with our previously reported results [22] as well as the reported findings by Larson *et al.*[11] who showed significant increases in histopathological lesion scores and the cell proliferation index in the liver of $B6C3F_1$ mice exposed to 90 ppm for 13 wk. A dose-related increase in the incidence of altered cell foci in the exposed female mice is causally related to the significant increase in the combined incidence of the hepatic tumors.

Rats: Exposure to chloroform induced nuclear enlargement of the proximal tubules and dilatation of the tubular lumen without development of renal tumors. A marked increase in the number of the exposed rats with urinary glucose can be taken to indicate lowered reabsorption of the glucose from the renal tubules, because the serum level of glucose remained normal. On the other hand, the BUN levels were lower in the exposed males than in the control. The incidence and severity of CPN tended to decrease with an increase in the exposure concentration. This tendency is consistent with the histopathological finding of the CPN observed in the chloroform-exposed F344 rats[12] and Osborne-Mendel rats[30], and could be explained in terms of the age-dependent increase in the CPN incidence and its decreased incidence due to suppression of the growth rate[27]. Lowered occurrence of myocardial fibrosis of the heart as an aging lesion was observed in the 90 ppm-exposed male rats.

As to the effects on the liver, no significantly increased incidence of the histopathological lesions was found even in the 90 ppm-exposed rats of both sexes except for the vacuolated cell foci. The serum biomarker levels of GOT, GPT and γ-GTP, indicative of liver cell necrosis and regeneration[31], tended to increase significantly but only slightly in the exposed male rats. The present finding of no renal cytoplasmic basophilia in the exposed rats is in contrast to the reported finding that inhalation exposure of F344 rats of both sexes to 90 ppm chloroform for 5 d/

wk and 13 wk produces fewer hyaline droplets and significantly enhanced cell proliferation in the kidneys, but no enhanced cell proliferation was found in the liver[12].

NOAELs for the occupational exposure limit

In the present study, a NOAEL was experimentally determined for the biologically significant endpoint at an exposure level above which a statistically increased incidence or biomarker level of the biologically significant endpoint occurred. For the exposed mice, the histopathological changes in the kidneys, including the cytoplasmic basophilia which occurred at 30 ppm and above, were employed as the biologically significant endpoint. The NOAEL value for the kidney endpoint resulted in 5 ppm for mice. For the exposed rats, the histopathological changes in the kidneys, including the nuclear enlargement of the proximal tubules and the dilatation of tubular lumen, both of which occurred at 30 ppm and above, were employed as the biologically significant endpoint, and the NOAEL value for the kidney endpoint resulted in 10 ppm. The slight but statistically significant increases in the serum levels of GPT and γ-GTP in the exposed male rats, which occurred at the lowest exposure concentration of 10 ppm, were not employed for a LOAEL for the liver endpoint, because the dose-response relationships for GPT and γ-GTP were not so unambiguous, although these two biomarkers are biologically significant, indicative of liver cell necrosis and regeneration[31].

The NOAELs determined in the present rodents study are in good agreement with those reported by Larson *et al.*[11] and Templin *et al.*[12] who determined a concentration of 10 ppm as the NOAEL for increased proliferation in the kidney proximal tubules of male and femaleF344 rats and in the liver of female mice. Therefore, the NOAEL of 5 ppm determined in the present mice study can be estimated as a threshold level above which the cytoplasmic basophilia in the kidney occurs as a regenerative, proliferative response to necrosis, leading to the development of renal cell adenomas and carcinomas through the pre-neoplastic atypical tubule hyperplasia based on the hypothesis of a non-genotoxic-cytotoxic mode of action for chloroform[7, 28, 29].

Comparison of the existing OEL value for chloroform among JSOH-OEL[4], ACGIH-TLV[5] and German MAK[32] indicates that the value of 10 ppm recommended by both ACGIH and JSOH is higher than that of 0.5 ppm set by the German MAK, although all three values are derived primarily from the bioassay data of rodents exposed to chloroform vapor by inhalation. The value of 10 ppm set by both ACGIH-TLV and JSOH-OEL was based on two separate studies showing that 7-h exposure of male rats to chloroform at 25–30 ppm for 6 months increased the relative liver and kidney weights[33, 34], whereas 4-h exposure to the same concentration for 6 months did not produce any liver lesion[34]. The OEL value of 10 ppm is presumed to be closely equivalent to the 4-h exposure of rats to 25 ppm[5]. On the other hand, the German MAK value of 0.5 ppm is based on a NOAEL of 5 ppm for an endpoint of increased cell proliferation in the liver and kidneys after inhalation of chloroform for 13 wk[32].

Assuming an uncertainty factor of 10 for extrapolating the rodent data to humans[35], one tenth of the NOAELs of 5 ppm for mice and 10 ppm for rats for the endpoint of renal histopathology may fall below the existing OEL value of 10 ppm in ACGIH-TLV and JSOH-OEL and be close to the German MAK value of 0.5 ppm. It is therefore suggested that the existing OEL values for chloroform be re-evaluated in light of the NOAELs determined chronically with mice and rats exposed to chloroform vapor for two years.

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