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Carcinogenicity of Sublimed Urethane in Mice through the Respiratory Tract

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The carcinogenicity of sublimed urethane (ethyl carbamate) in air was examined with mice. JCL:ICR mice were nursed in a plastic cage inside a vinyl chamber which was ventilated 4 times per hour. The mice were exposed to urethane gas for various periods by passing air which contained a high concentration of sublimed urethane (1.29 μg/ml) into the vinyl chamber, or by placing a vessel containing crystalline urethane inside the vinyl chamber so that it was filled with spontaneously-sublimed urethane gas at a low concentration (0.25 μg/ml). When female mice were killed 5 months after exposure, lung tumor frequency increased almost linearly with the number of days of exposure in the low concentration experiment, but increased in a non-linear manner in the high concentration experiment. In terms of nearly the same total dose, i.e., (concentration of urethane gas in air)×(days of inhalation), one day of exposure to urethane gas at the low concentration induced lung tumors at a significantly higher frequency than 1/4 day of exposure to urethane gas at the high concentration. When male mice were killed at 12 months after exposure to examine the progressive change of induced tumors, malignant, invasive and metastatic tumors were found to have been induced more frequently in the lung after exposure to urethane gas at the low concentration (0.25 μg/ml for 10 days) than at the high concentration (1.29 μg/ml for 4 days), although the total dose in the former group was about half of that in the latter. Continuous exposure to urethane gas for a longer period at the low concentration seems to be more efficient for the induction, promotion and/or progression of lung tumors than the exposure for a shorter period at the high concentration.

Key words: Urethane gas — Sublimation — Carcinogenicity — Dose rate — Mice

Urethane has long been used as a solvent for pesticides, cosmetics and more than 200 parenteral drugs,[1,2] and small amounts of urethane are formed spontaneously in Japanese sake, wine, whisky, brandy, and fermented beverages and foods.[3,4] Furthermore, it is volatile and has proven to be useful in the formation of enormous numbers of products in every-day use.[1,5–10] Even from crystalline urethane, a small amount spontaneously sublimed and induced a high frequency of recessive lethal mutations in *Drosophila melanogaster*.[5] Consequently, urethane could be a hazardous carcinogen to which the human respiratory tract is exposed.

In this paper, the carcinogenicity of sublimed urethane gas was examined quantitatively in mice treated with various concentrations for various periods. Lung tumors were assayed, because they are suitable for quantitative analysis[1,11–13] and the lung tissue is the target of toxic gases.

MATERIALS AND METHODS

Animals JCL:ICR mice (28 days old and weighing 18–22 g) were purchased from CLEA Japan Co., Ltd. (Ishibe). Mice were maintained under SPF conditions with mouse diet CRF-1 (Charles River Japan, Kanagawa) and chlorinated tap water at 23–25°C.

Urethane gas inhalation The experimental procedure is shown schematically in Fig. 1. Filtered air at 25 °C was passed into the vinyl chamber (45 liter) by a booster fan (Model NM, Moriyasu Co. Ltd., Osaka) at the rate of 3 liter/min to ventilate the chamber about 4 times per hour. In each experiment, 20 to 25 mice were nursed in a plastic cage (20×32 cm, 13 cm depth) inside the vinyl chamber. To provide urethane gas at the high concentration (Fig. 1-A), air was passed first through a glass bottle containing 500 g of crystalline urethane (ethyl carbamate, Wako Pure Chemical Ind. Ltd., Osaka) and then into the vinyl chamber. Mice in the chamber thus inhaled air saturated with urethane gas. To provide urethane gas at the low concentration (Fig. 1-B), 20 g of crystalline urethane was placed in a vessel (10×20 cm, 3 cm depth) beside the mouse cage. Urethane sublimed spontaneously, and gaseous urethane was therefore present in the vinyl chamber. The concentration of urethane gas in the chamber was estimated by means of the formula, $Wo - We/V$, where Wo and We are the weight of urethane at the beginning and the end of the experiment, respectively, and V is the total volume of ventilated air during the experiment. The concentrations (mean ±

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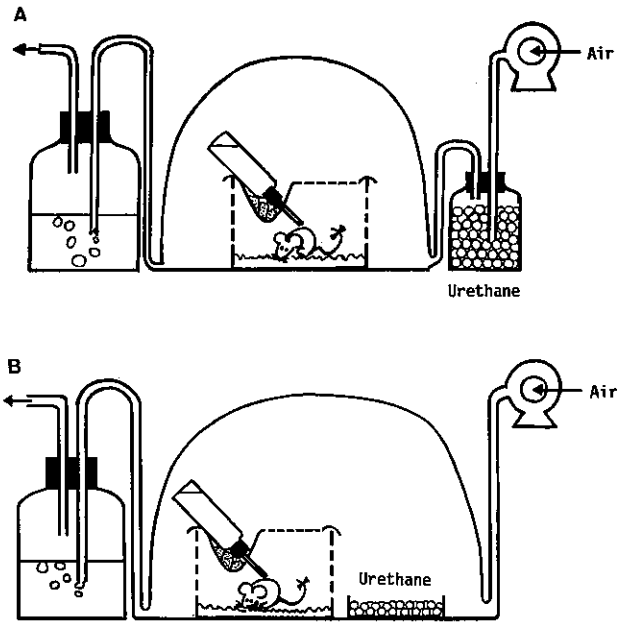

Fig. 1. Scheme of experimental procedure for urethane gas inhalation. Details are given under "Materials and Methods."

95% confidence interval) were 1.29 ± 0.14 μg/ml, n=55 (353 ± 37 ppm) and 0.25 ± 0.04 μg/ml, n=10 (68 ± 12 ppm) for the high and low concentration experiments, respectively. Female JCL:ICR mice inhaled a high or low concentration of urethane gas for 0.25, 1, 2, 4, 5 and 7 days, or 1, 3, 5, and 10 days, respectively. Male JCL:ICR mice were also treated for 4 or 10 days with a high or low concentration of urethane gas, respectively.

Examination of tumors Female mice were killed by cervical dislocation at 5 months after treatment. Gross pathological lesions, tumors especially, were examined as described previously,[1, 12] and specimens were microscopically examined.[12] Lung tumors, lymphocytic leukemias and uterine hemangiomas were the predominant types of induced tumors. Lung tumors in mice possess various advantages in dose-response experiments, as follows: (a) these tumors are induced by almost all carcinogens,[12, 13] (b) the number of tumors reaches a maximum approximately 10 weeks after treatment, and then remains at that level, while the size and histological patterns of tumors change as time passes,[12] (c) tumors can be evaluated quantitatively (as in the case of counting bacterial colonies), that is, lung tumor frequency defined as the average number of tumor nodules per lung increases almost linearly with increasing dose of chemical carcinogens,[1, 12] while lung tumor incidence defined as percent of tumor-bearing mice per survivor levels off at higher doses.[1, 12, 13] When treated mice were observed for longer than 5 months after treatment, tumor nodules fused to each other and became difficult to count accurately. Finally, (d) lung tumors are transformed into malignant tumors.[11–13] Histological classification of lung tumors was conducted as in our previous studies.[11, 12] To examine such progressive changes of induced lung tumors and others (e.g., hepatomas), male mice were observed for a longer period and killed at 12 months after exposure.

RESULTS

Acute toxicity of sublimed urethane gas When mice inhaled the high concentration of urethane gas for 4, 5 and 7 days, 14.9% (15 of 101 females and 7 of 47 males), the 28.0% (7 of 25 females), and 75% (9 of 12 females) of the mice died of acute toxicity within 7 days after the end of exposure, respectively. Such acute death was not observed in other experimental groups of mice which were exposed to the high concentration of urethane gas for shorter periods or to the low concentration for 1 to 10 days. Microscopically, hemorrhagic pneumonitis was observed in all dead mice.

Carcinogenicity of urethane gas at high and low concentrations Table I shows the incidence of tumors produced in females at 5 months post-treatment. When female mice were exposed to the high concentration of urethane gas, about half of them developed lung tumors or lymphocytic leukemias with 6 h inhalation and the inidence reached maximum with 24 h inhalation. However, the frequency of lung tumors decreased when mice were exposed to the high concentration of urethane gas for longer periods (4 and 5 days). The low concentration of urethane gas also produced significant yields of lung tumors and leukemias. Exposure for one day induced tumors in a half of the mice. In the low concentration experiment, lung tumor frequency increased almost linearly with days of exposure. When the dose-response relationships were compared on the basis of the total dose, i.e., (concentration of urethane gas in air) × (days of inhalation), there was no substantial difference in the lung tumor frequency between the low and high concentration experiments in the range of the total doses from 1.25 to 2.58 (μg/ml·days). At the low total dose of about 0.3 (μg/ml·days), however, the low concentration experiment showed a significantly higher frequency of lung tumors than the high concentration experiment ($P < 0.05$ in Table I).

When male mice were killed 12 months after treatment to examine the progression of induced lung tumors, exposure to urethane gas at the low concentration (0.25 μg/ml) for a longer period (10 days) gave a significantly higher incidence of malignant and invasive tumors in the

Table I. Incidence of Tumors in Female JCL:ICR Mice Killed 5 Months after Inhalation of Sublimed Urethane Gas

Concentration in air (μg/ml)	Days of inhalation	Total dose (μg/ml·days)	Lung tumor		Leukemia	Others[a]
			Incidence (%)	Tumors/lung	Incidence (%)	
1.29	0.25	0.32	38/79 (48.1)	0.67±0.20[b],*	2/79 (2.5)	
	1	1.29	37/40 (92.5)	10.7±2.9	1/40 (2.5)	1 UH
	2	2.58	66/70 (94.3)	18.6±3.8	12/70 (17.1)	2 UH, 1 A
	4	5.16	81/86 (94.2)	10.6±2.6	18/86 (20.9)	8 UH, 1 BC
	5	6.45	18/18 (100)	12.2±3.9	3/18 (16.7)	
0.25	1	0.25	27/51 (52.9)	1.08±0.39*	3/51 (5.9)	
	3	0.75	44/51 (86.3)	5.29±1.28	2/51 (3.9)	1 UH
	5	1.25	46/53 (86.8)	7.56±2.05	5/53 (9.4)	1 RS
	10	2.5	9/11 (81.8)	17.8±4.6	0/11 (0.0)	
0[c]		0	2/51 (3.9)	0.04	0/51 (0.0)	
Control[d]		0	8/198 (4.0)	0.04±0.03	0/198 (0.0)	

a) UH, uterine hemangioma; A, aneurysm of thoracic aorta; BC, breast cancer; RS, reticulum cell neoplasia.
b) Mean and 95% confidence limit of the mean computed from *t*-distribution.
c) Concurrent controls nursed in the vinyl house for 10 days without urethane inhalation.
d) Untreated controls pooled during the experiments.
* $P<0.05$. The *t* test was applied after testing the variance ratio.

Table II. Incidence of Tumors in Male JCL:ICR Mice Killed 12 Months after Inhalation of Sublimed Urethane Gas at Low or High Concentration

Concentration in air (μg/ml)	Days of inhalation	Total dose (μg/ml·days)	No. of mice	Lung tumors				Leukemia		Hepatoma		Others	
				Adeno-carcinoma		Invasion or metastasis							
				No.[a]	(%)	No.[b]	(%)	No.	(%)	No.	(%)	No.	(%)
0	0	0	51	1	(2.0)	0	(0.0)	0	(0.0)	1	(2.0)	0	(0.0)
0.25	10	2.5	50	40*	(80.0)	11*	(22.0)	5	(10.0)	6	(12.0)	3[c]	(6.0)
1.29	4	5.16	40[d]	14	(35.0)	0	(0.0)	8	(20.0)	3	(7.5)	0	(0.0)

a) No. of mice having at least one adenocarcinoma in the lung.
b) No. of mice having invasion or metastasis of lung tumors to thoracic wall, spleen, and lymph nodes.
c) One adenocarcinoma of the pancreas with bloody ascites and metastasis to diaphragm; one subcutaneous fibrosarcoma, and one subcutaneous hemangioendothelioma.
d) Seven of 47 males died within 7 days after treatment.
* $P<0.01$ by χ^2-test with Yates' correction vs. high concentration exposure.

lung than exposure at the high concentration (1.29 μg/ml) for a shorter period (4 days) ($P<0.01$ in Table II), although the total dose in the former group was about half of that in the latter goup. Some tumors grew to large sizes (5 to 10 mm), invaded adjacent organs, and metastasized to hilar, mediastinal and intercostal lymph nodes, and spleen. Hepatomas and other malignant tumors were also induced in a higher incidence after exposure to the low concentration of urethane gas for the longer period, while the incidence of lymphocytic leukemias increased in parallel with the total dose.

DISCUSSION

This is the first report showing that inhaled urethane gas induced malignant tumors in mice. Lung tumor frequency in mice showed an approximately linear increase with respect to the total dose of sublimed urethane gas, i.e., (concentration in air)×(exposed days) for exposure at the concentration of 0.25 μg/ml in air, whereas inhalation of urethane gas at the concentration of 1.29 μg/ml resulted in a complicated total dose-response relationship with a maximum at 2.5 (μg/ml·days) and a

decreasing trend at higher doses. Degenerative changes were induced in the lung (and probably in other organs) by the over-doses, i.e., longer exposures at the high concentration, and these may have caused the reduction of tumor yields in these experimental groups. At a roughly equal total dose of 0.3 (μg/ml·days), the exposure to urethane gas at the low concentration gave rise to a significantly higher frequency of lung tumors than that at the high concentration (Table I). Similarly, induced tumors were more malignant and more metastatic after exposure to urethane gas at the low concentration than at the high concentration (Table II).

These results are contrary to expectation based on the assumption that urethane initiates cancer by inducing tumorigenic mutation via action on DNA, because mutagenic action of a carcinogen usually decreases with decrease in the administration rate of the carcinogen. In fact, the yields of both mutations and tumors induced by ionizing radiation in mice are reduced by lowering the dose-rate.[14–18] Thus, it is tempting to speculate that the major tumorigenic action of urethane is not the initiation of tumorigenesis by inducing mutations, but rather the subsequent promotion and progression of induced events. In fact, urethane possesses a potent tumor-promoting action[19, 20] in comparison with its mutagenicity in mice.[20]

Even for radiation, a well-known potent mutagen, evidence is available to show that it acts as a promoter and/or a progressor of tumors. Lymphocytic leukemia in the thymus is more frequently induced by four fractionated whole-body X-irradiations of mice with moderate doses (1.61 Gy per exposure) at weekly intervals than by a single irradiation with the same total dose[21]; this tumor is known to be induced indirectly by the radiation-induced damage to the host animal rather than direct action on the target cells in the thymus.[22, 23] Similarly, the incidence of skin tumors and osteosarcomas appeared to be determined by the total number of repeated exposures in the moderate dose range (2.5 to 3.5 Gy) rather than the total doses,[24, 25] and a single irradiation was inefficient.[24] In the present study, continuous exposure to urethane gas for a longer period at the low concentration of 0.25 μg/ml may have been optimum for the promotion and/or progression of lung tumors, as more malignant, invasive, and metastatic tumors were observed than in the case of the exposure at 1.29 μg/ml for a shorter period.

This work should serve to alert health agencies, because urethane is volatile and is employed for various industrial purposes.[5–10] It is difficult to perceive and avoid chronic exposure to low doses of toxic gases through the respiratory tract; the carcinogenic potential of such exposures is demonstrated in the present study.

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