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Quantitative Risk Assessment of Carcinogenicity of Urethane (Ethyl Carbamate) on the Basis of Long-term Oral Administration to B6C3F$_1$ Mice

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A carcinogenicity study of urethane was performed for quantitative assessment of its risk in humans. Three hundred 6-week-old male B6C3F$_1$ mice were divided into 6 groups, each consisting of 50 mice, and urethane was given *ad libitum* in drinking water at levels of 0 (control), 0.6, 3, 6, 60 and 600 ppm for 70 weeks. The tumors with a clear dose-response relationship were lung tumor (alveolar/bronchiolar adenoma or carcinoma) and liver tumor (hemangioma or angiosarcoma). The incidences of these two types of tumor were applied to estimation of the virtually safe dose (VSD) at the level of 10^{-6} by using four mathematical models (Logit, Probit, Weibull and Multistage models). The VSD based on the incidences of lung tumor by using the Logit model was estimated to be 1.8×10^{-4} mg/kg body weight/day. On the other hand the VSD based on those of liver tumor by using the Weibull model was 7.2×10^{-5} mg/kg body weight/day. Thus, the VSDs based on the incidences of the two different types of tumor using the most compatible mathematical model in each case, as judged from the *P*-values, were similar.

Key words: Urethane — B6C3F$_1$ mouse — Carcinogenicity — Virtually safe dose

Urethane, ethyl carbamate, was formerly used as an antineoplastic agent, in the treatment of chronic leukemia and multiple myeloma. At present its major use is as a chemical intermediate useful as a cross-linking agent in textile treatments and also as an anesthetic for laboratory animals.[1]

Since Nettleship *et al.*[2] first indicated the carcinogenicity of urethane in mice, extensive carcinogenicity studies using several species of experimental animals have been carried out, and urethane has been shown to be carcinogenic in mice, rats and hamsters following administration by the oral, inhalation, subcutaneous and intraperitoneal routes.[1] A general review has been published by Mirvish.[3]

No case report or epidemiological study regarding the carcinogenicity of urethane in humans is available,[1] but it was announced in 1985 that relatively high levels of urethane were present in certain types of wines and other alcoholic beverages in Canada.[4] While it is recognized that urethane can occur naturally at very low levels in alcoholic beverages as a result of the fermentation process,[5] the levels in some products were clearly in excess of naturally occurring amounts. The source of the elevated level of urethane was attributed to the reaction between urea and ethanol.[4] Thus, it is desirable to carry out a risk assessment of urethane carcinogenicity to establish a guideline level as a limit of exposure to urethane in humans.

The present study was conducted, therefore, to obtain dose-response data of tumor incidence in mice administered urethane orally and to make an estimation of the virtually safe dose (VSD), defined as a value corresponding to the dose level which can induce tumors at extremely low rates, such as 10^{-6} or 10^{-8}.[6–11] This value can be obtained by downward extrapolation (low-dose extrapolation) of dose-response data of tumor incidence in animals using a compatible mathematical model.

MATERIALS AND METHODS

Experiment Three hundred male B6C3F$_1$ mice at the age of 4 weeks were purchased from Charles River Japan Inc. (Atsugi, Kanagawa). After a 2-week quarantine period, the 6-week-old mice were divided into 6 groups, each consisting of 50 mice. Mice in the five treated groups were given *ad libitum* drinking water containing urethane at levels of 0.6, 3, 6, 60 and 600 ppm for 70 consecutive weeks. The urethane utilized in the present study had a purity of more than 99% (Kanto Chem. Indust. Co., Tokyo) and a fresh solution using distilled water was prepared every 3 days. Mice in the control group were given distilled water and all mice, both treated and control, had access *ad libitum* to a basal diet (CRF-1, Charles River Japan, Inc.). All mice were housed, five to a plastic cage, and all cages were kept in the same air-conditioned animal room. The room temperature and humidity ranged from 23°C to 26°C and from 60% to 70%, respectively.

At the end of the 70-week treatment period all surviving mice were anesthetized with ether and autopsied.

Any mouse found dead or moribund during the treatment period was autopsied. At autopsy all major organs were weighed and all organs or tissues and tumor masses required for the carcinogenicity study were prepared for microscopic examination.

Amounts of drinking water consumed per cage over 3 consecutive days were measured once a week during the treatment period. Each mouse was weighed once every 2 weeks until the 14th week and thereafter once every 4 weeks.

Risk assessment Statistical analysis of differences in tumor incidence was performed by using the chi-square test and that of differences in mean values for survival time was done by using Student's t test. For risk assessment, four well-known mathematical models, i.e., the Logit, Probit, Weibull and Multistage model were used to obtain the values of VSD at the level of 10^{-6}. Each of the models shows a different dose-response relationship at the low-dose level according to a different mathematical curve. For example, the relationship is linear in the Multistage model, but sigmoid in the Probit model.

RESULTS

Survivals of mice All of the mice in the 600 ppm group died by the 46th treatment week, while in the other groups a few mice died after the 24th week. The mean survival times are shown in Table I and no significant difference in the mean survival times between the control group and the treated groups was noted except for the 600 ppm group.

Mice were regarded as "effective" for data analysis if they survived beyond the 23rd week, the time at which the first death with a lung tumor occurred in the 600 ppm group. As shown in Table I, more than 96% of the mice were "effective" in all groups except for the 600 ppm group. In that group one mouse died with malignant lymphoma at the 17th week and others had to be excluded owing to insufficient histological examination, although they died during the period from the 29th week to the 36th week.

Daily intakes of urethane by mice The mean values of daily intakes of urethane per mouse, which were estimated from the measured amounts of drinking water are also shown in Table I. There was no significant difference in the amounts of drinking water among the groups, and therefore the ratios of daily intake of urethane adjusted for body weight of mice among the groups took the values designed initially.

Tumor incidences in mice The numbers of mice with various histological types of tumors including two types of tumor-related lesions are shown in Table II. The tumor with the highest occurrence was alveolar/bronchiolar adenoma or carcinoma of the lung, and furthermore, the incidences revealed a dose-response relationship in the treated groups. All of the mice with alveolar/bronchiolar carcinoma belonged to the 600 ppm group and the proportion of mice with multiple adenomas was extremely high in the 60 ppm and 600 ppm groups, as shown in Table III.

Hemangioma or angiosarcoma of the liver was the tumor with the second-highest occurrence and the incidences also revealed a dose-response relationship in the treated groups. The death of mice in the 600 ppm group during the early experimental period was mostly attributed to the rupture of hepatic capsule overlying this type of liver tumor. Peliosis, a non-neoplastic lesion involving dilated sinusoids, was frequently seen in the 60 ppm and 600 ppm groups and the incidences as well as histological findings suggested that it was a hemangioma-related lesion of a preneoplastic lesion of hemangioma.

The incidences of hepatocellular adenoma or carcinoma varied among the treated and the control groups and no dose-response relationship was noted. Other types of tumor were occasionally seen, but there was no significant evidence of carcinogenesis by urethane.

Application of mathematical models to tumor incidences for risk assessment of carcinogenicity Based on the

Table I. Number and Mean Survival Time of Effective Mice and Mean Daily Intake of Urethane

Dose (ppm)	No. of mice		Mean survival time (range) (week)	Mean intake of urethane μg (mg/kg body weight)
	Initial	Effective		
0	50	49	69.5±2.4 (57–70)	0
0.6	50	49	69.7±2.0 (56–70)	3 (0.095)
3	50	48	65.5±9.3 (24–70)	19 (0.58)
6	50	50	69.8±1.4 (60–70)	33 (1.0)
60	50	50	66.9±8.5 (24–70)	320 (10)
600	50	44	39.2±5.0* (23–46)	3200 (100)

* Significantly different from the 0 ppm group by t test ($P<0.01$).

Table II. Incidence of Mice with Various Histological Types of Tumors and Tumor-related Lesions

Sites	Histological types of tumors and tumor-related lesions	No. and incidences (%) of mice with tumors					
		Urethane dose (ppm) of:					
		0	0.6	3	6	60	600
Lung	Alveolar/bronchiolar adenoma	9 (18.4)	4 (8.2)	7 (14.6)	8 (16)	34 (68)*	42 (95.5)*
	Alveolar/bronchiolar carcinoma	0	0	0	0	0	6 (13.6)*
Liver	Foci of cellular alteration[a]	1 (2.0)	5 (10.2)	3 (6.3)	1 (2)	5 (10)	0
	Hepatocellular adenoma	8 (16.3)	2 (4.1)	8 (16.7)	4 (8)	9 (18)	0
	Hepatocellular carcinoma	0	2 (4.1)	1 (2.1)	0	2 (4)	0
	Peliosis[a]	0	0	0	0	7 (14)*	4 (9.1)**
	Hemangioma	0	0	0	0	2 (4)	20 (45.5)*
	Angiosarcoma	0	0	0	2 (4)	2 (4)	11 (25)*
RES	Lymphoma	0	0	1 (2.1)	0	0	1 (2.3)
Soft tissue	Malignant fibrous histiocytoma	1 (2.0)	0	0	0	0	0
Spleen	Hemangioma	0	1 (2.0)	0	1 (2)	2 (4)	0
Stomach	Papilloma	1 (2.0)	0	0	0	0	0
	Squamous cell carcinoma	0	0	0	0	1 (2)	0
Pancreas	Hemangioma	0	0	0	0	1 (2)	0
Heart	Hemangioma	0	0	0	0	0	4 (9.1)**

a) Tumor-related lesions.
* Significantly different from the 0 ppm group by chi-square test ($P<0.01$).
** Significantly different from the 0 ppm group by chi-square test ($P<0.05$).

Table III. Incidence of Mice with Lung Tumor

Dose (ppm)	No. of effective mice	No. of mice with lung tumor (%)	No. of mice with adenoma (%)				No. of mice with carcinoma
			No. of adenoma				
			1	2–5	6–10	10<	
0	49	9 (18.4)	7 (14.3)	2 (4.1)	0	0	0
0.6	49	4 (8.2)	3 (6.1)	1 (2.0)	0	0	0
3	48	7 (14.6)	5 (10.4)	2 (4.2)	0	0	0
6	50	8 (16)	7 (14)	1 (2)	0	0	0
60	50	34 (68)	10 (20)	23 (46)	1 (2)	0	0
600	44	42 (95.5)	4 (9.1)	15 (34.1)	5 (11.4)	18 (40.9)	6 (13.6)

Table IV. Estimated Virtually Safe Doses (VSDs) for Lung Tumor and Liver Tumor in B6C3F$_1$ Mice at the Level of 10^{-6}

Tumor site	Mathematical model			
	Logit model	Probit model	Weibull model	Multistage model
Lung				
VSD[a]	1.8×10^{-4}	9.2×10^{-3}	8.4×10^{-8}	7.3×10^{-5}[b]
P-value	0.3261	0.2942	0.0229	0.3257[b]
Chi-square value	3.46	3.71	9.54	2.24[b]
Liver				
VSD[a]	3.6×10^{-4}	1.9×10^{-2}	7.2×10^{-5}	9.6×10^{-5}
P-value	0.1966	0.0693	0.3878	0.1954
Chi-square value	6.03	8.69	4.14	4.70

a) mg/kg body wt/day.
b) These values were calculated excluding the data from the 600 ppm group.

results in the present experiment, the incidences of lung tumor (alveolar/bronchiolar adenoma or carcinoma) and liver tumor (hemangioma or angiosarcoma) revealed a clear dose-response relationship, and therefore, the incidences of these two types of tumor could be employed in the estimation of VSD at the level of 10^{-6} by the use of four mathematical models.

The values of VSD as well as *P*-values and chi-square values, which are indicators of the compatibility of models, are shown in Table IV. The Logit model was the most compatible with the incidences of lung tumor as judged from the *P*-value as well as the chi-square value and the VSD according to the Logit model was estimated to be 1.8×10^{-4} mg/kg body weight/day. On the other hand, the Weibull model was the most compatible with the incidences of liver tumor and the VSD by using the Weibull model was estimated to be 7.2×10^{-5} mg/kg

body weight/day. The values based on the incidences of these two different types of tumor by using the most compatible mathematical models were similar.

DISCUSSION

Based on our historical data,[12-15] the spontaneous tumors with relatively high occurrence in male B6C3F$_1$ mice are hepatocellular tumor (adenoma or carcinoma) of the liver, alveolar/bronchiolar tumor (adenoma or carcinoma) of the lung and lymphoma/leukemia. Hemangioma of the liver is occasionally seen but angiosarcoma is very rare. The organ distribution of tumors observed in the control group of the present study was similar to those in our historical data, but the incidences of tumors were generally low because of the shorter experimental period in the present study than those in our historical data. For example, the incidences of hepatocellular adenoma of the liver, 16.3% and hepatocellular carcinoma of the liver, zero in the present study were lower than those in our historical data, that is, 19%[13] or 22%[15] of adenoma and 22%[13] or 13%[15] of carcinoma, respectively. The incidence of lymphoma/leukemia in the present study was zero, while it was 19%[13] or 11%[15] in our historical data.

As for alveolar/bronchiolar adenoma or carcinoma of the lung, the spontaneous tumor incidences in B6C3F$_1$ male mice were 5%,[12] 16%,[13] 11%[14] or 4%[15] in our historical data and therefore, high incidences in the 60 ppm and 600 ppm groups associated with a clear dose-response relationship were indicative of carcinogenicity of urethane. It might be supposed that the higher incidence in the control group than those in the 0.6, 3 and 6 ppm groups indicated a suppressive effect of urethane on carcinogenesis in the lung, but compared with our historical data mentioned above, the high incidence in the control group could be regarded as fortuitous. On the other hand, hemangioma or angiosarcoma of the liver occurred spontaneously at the rates of 8%,[12] 13%[13] or 0%[14, 15] in our historical data, and therefore, the high incidence of hemangioma, 45.5% and that of angiosarcoma, 25% in the 600 ppm group were also indicative of carcinogenicity of urethane. We consider that these results confirm the carcinogenicity of oral urethane in B6C3F$_1$ mice, and show that the target organs of urethane were lung and liver.

Some of the carcinogenicity studies previously reported can be used for risk assessment of orally administered urethane,[16-21] and the study reported by Schmähl *et al.*[21] was considered to be the most suitable because of a clear dose-response relationship of tumor incidences. In that study, NMRI mice at the age of 8 weeks were used and fed for 660 days in the daily dose group of 12,500 μg/kg body weight, 730 days in the 2500, 500 and 100 μg/kg body weight groups and 760 days in the control group. It was reported that lung tumor (mainly adenoma and carcinoma), malignant mammary tumor and hemangioendothelioma (mainly in the liver) showed incidences with a clear dose-response relationship, though the table shown in that report gave the overall incidences of malignant tumor or leukemia. Therefore, if VSD is estimated from the data reported by Schmähl *et al.*, these overall incidences including the incidences of various histological types of tumor have to be used. Thus, by using the Weibull model the VSD at the 10^{-6} level was estimated to be 5.8×10^{-16} mg/kg, which is extremely different from the VSD based on the results in the present study. One of the reasons for the extreme difference is presumably the tumor incidences used in the estimation. In the present study the incidences of a specific type of tumor were used for estimation of VSD, whereas the overall incidences of malignant tumor were used in Schmähl's study.[21] In addition, the high-dose group in Schmähl's study showed lower tumor incidences than that in the present study, although the tumor incidences of the low-dose groups were similar, because the incidences of lung and liver tumor in the present study included benign tumor.

In Canada, as part of the procedure for establishment of guidelines to limit urethane levels in alcoholic beverages, Schmähl's study was reviewed.[4] The no-observed-effect levels (NOEL) for rodents was estimated to be of the order of 1500 μg/kg body weight, and a safety factor of 5000 was applied to this NOEL to estimate a tolerable daily intake (TDI) for humans of 0.3 μg/kg body weight. Furthermore, the average TD_{50} for 45 different tumors in 4 species (mouse, rat, hamster and monkey) from the results of seven studies was judged to be 130 mg/kg body weight and the average TD_1 was calculated to be 2.6 mg/kg body weight. On the basis of these findings the VSD at the 10^{-6} level was determined to be 0.26 μg/kg body weight and the maximum tolerable daily intake of urethane in humans was estimated to be 0.3 μg/kg body weight. In view of the estimated mean daily intake by the portion of the population which consumes alcoholic beverages, the following guidelines were established to limit urethane in alcoholic beverages: table wine 30 ppb, fortified wine 100 ppb, distilled spirits 150 ppb, fruit brandies 400 ppb.[4]

The VSD at the 10^{-6} level estimated in Canada, 0.3 μg/kg body weight, is similar to the VSD, 0.18 μg/kg body weight, estimated based on the incidences of lung tumor in the present study. Consequently it is thought that the results in the present study well support the guidelines to limit urethane in alcoholic beverages in Canada.[4]

In the estimation of VSD in the present study using four mathematical models, the Logit, Probit, Weibull

and Multistage models, the *P*-values, which indicate the compatibility of the models, were smaller than that in a previous report.[11] This may suggest that none of the models for estimation of VSD is suitable for the data on tumor incidences obtained in the present study. However no definite criteria have been established for the selection of a mathematical model,[22] and therefore, it appears at present that the estimation of VSD in the present study should be carried out using the model with the largest *P*-value.

The selected model for lung tumor was the Logit model and that for liver tumor was the Weibull model in the present study. Although the selected model was different between the two types of tumors, the estimated VSDs were similar. The results may indicate that when two different types of tumor showing incidences with a clear dose-dependent relationship are induced in a certain carcinogenicity study, the incidences of the different types of tumor are able to lead to similar VSDs if the most suitable mathematical model is used for estimation in each case. Further investigations to examine the validity of this speculation seem worthwhile.

Urethane has been shown to be mutagenic, teratogenic and carcinogenic, though there is some evidence to suggest that urethane *per se* is not carcinogenic but requires metabolic activation to exert its carcinogenic action.[1] Any mathematical model is dependent on an interpretation of the carcinogenic process, although no principle for model selection has been established. Accordingly, it can not be determined which model should be selected for estimation of VSD of chemicals from the viewpoint of the carcinogenic process. Further investigations are clearly necessary.

The reported VSDs of well known carcinogens obtained by using the Weibull model are as follows: dimethylnitrosoamine, 1.9×10^{-2} ppm, ethylnitrosourea, 5.65×10^{-6} ppm, aflatoxin B_1, 4.0×10^{-2} ppb.[23] The VSD of urethane, 7.2×10^{-5} mg/kg (based on the incidences of liver tumor in the present study analyzed by using the Weibull model) is 4.6×10^{-4} ppm as a concentration in drinking water. Thus, the carcinogenicity of urethane is thought to be intermediate between those of dimethylnitrosamine and ethylnitrosamine. Environmental chemicals have been suspected of being the most important cause of human cancers, and so it is important to carry out quantitative estimation of the carcinogenicity of chemicals, e.g. by determination of VSD based on experimental studies. The limitation of use of chemicals on the basis of VSD data might be able to play a major role in the primary prevention of human cancer.

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