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MULTICELLULAR IN VIVO SISTER-CHROMATID EXCHANGES INDUCED BY URETHANE

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Summary

Following urethane inhalation exposure, clear dose—response relationships were apparent in all cell types examined in hepatectomized and intact mice. At concentrations of 0.1 mg/l and higher, induced SCE frequencies were linearly related to log urethane concentrations. No significant differences in SCE response between like cell types of hepatectomized and intact mice were apparent. In hepatectomized mice, there was no significant difference in the SCE response of regenerating liver and alveolar macrophage cells. However, bone-marrow response was significantly lower ($p = 0.01$). Likewise, in intact mice bone marrow response was significantly lower than in alveolar macrophages ($p = 0.01$).

Inhalation and intravenous infusions of the same total dose of urethane (193 mg/kg) administered over a 4-h period produced comparable SCE responses in all cell types. However, a single intraperitoneal injection of 193 mg/kg just prior to BrdU infusion produced significantly higher SCE frequencies in bone marrow ($\alpha = 0.01$), and alveolar macrophages ($\alpha = 0.05$) of intact mice than did the equivalent inhalation dose. Intraperitoneal injections produced similar results in 2-month-old mice as in 4-month-old mice. However, regardless of the route of administration SCE frequencies in regenerating liver and/or alveolar macrophages were significantly higher than in bone marrow.

We have recently reported an in vivo simultaneous multicellular sister-chromatid exchange (SCE) assay utilizing murine alveolar macrophages, regenerating liver and bone-marrow cells (Conner et al., 1979a). Using the described assay, we observed dose-dependent increases in SCE frequencies in all cell types

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following inhalation exposures of mice to styrene but no tissue-specific differences in cellular SCE responses were apparent over all concentrations employed (Conner et al., 1979b, 1980). In order to investigate whether the multicellular assay might be sensitive to tissue-specific effects, we evaluated relative multicellular SCE induction by the extensively studied murine mutagen/carcinogen, urethane (ethyl carbamate).

Regardless of its route of administration, urethane is rapidly and uniformly distributed throughout all major body organs of fetal, young and adult mice (Nomura, 1976; Prodi et al., 1970). Despite such uniformity, urethane is generally associated with production of alveolar adenomas irrespective of age or sex of mice (Pound and Lawson, 1974; Nomura, 1976). In addition, young mice as well as hepatectomized male mice demonstrate a sensitivity to urethane-induced hepatomas (Hollander and Bentvelzen, 1968; Chavan and Bhide, 1972; Prodi et al., 1970; Pound and Lawson, 1974; Nery, 1968; Chernozemski and Warwick, 1970).

Recently, urethane and its presumably active metabolite, hydroxyurethane, were found to produce dose-dependent increases in SCE frequencies in cultured human lymphocytes (Csukas et al., 1979). Just as the uniform tissue distribution of urethane makes it possible to quantitatively compare relative tumor susceptibilities of various organs in mice, it was anticipated that similar multicellular comparisons might be made regarding urethane-induced SCE in vivo. In the present study, we have found that inhalation of urethane aerosols produces dose-dependent SCE responses in alveolar macrophages, regenerating liver and bone-marrow cells of hepatectomized mice and in bone marrow and alveolar macrophage cells of intact mice. Furthermore, the same relative tissue specificities were observed for urethane-induced SCE as previously reported for tumor induction.

Materials and methods

4-month-old BDF_1 male mice were used throughout this study. In addition, 2-month-old BDF_1 males were used in the intraperitoneal injection studies of intact mice. For liver studies, mice were subject to partial hepatectomy as previously described (Conner et al., 1978) between 11:00 a.m. and noon 5 days prior to urethane treatment. Urethane (99% purity) was purchased from Aldrich.

Inhalation exposures

A 2.5-l glass exposure chamber was used (Barrow et al., 1977), and operated at 20 l/min. 4 mice (2 hepatectomized and 2 intact) were exposed at a time by head-only exposure for the duration of 4 h (11:15 a.m.—3:15 p.m.) on the 5th day post-hepatectomy. Each exposure concentration was repeated once in order to obtain 4 hepatectomized and 4 intact mice at each concentration level. All experimental groups included non-exposed controls.

Urethane solutions of concentrations (%, w/v) 0.5, 1.6, 2.5, 5.0, 10.0, and 20.0 were prepared. These solutions were aerosolized by a glass Dautrebande generator to obtain nominal chamber concentrations of 0.035, 0.11, 0.22, 0.475, 0.90 and 2.06 mg/l resp. To verify these concentrations, samples were

taken from the exposure chambers on polytetrafluoroethylene filters at a sampling rate of 1 l/min. However, extensive sublimation made it impossible to verify the concentration by this direct method. Instead a tracer technique was used, adding 0.0074% (w/v) of uranine to each of the above solutions to calibrate the generator output by fluorescence analysis of uranine content of filter samples taken from the exposure chamber. The results of 5 determinations indicated an agreement within 10% between the nominal concentrations given above and the concentrations found with the tracer technique. Particle size analysis with a TSI Whitby Analyzer using a 0.1% uranine solution revealed a mass median diameter (MMD) of 0.2 μm with a geometric standard deviation of 1.6 and the parent droplet being 2.0 μm. This is in agreement with a 1.7-μm value for the parent droplet cited in the literature for this type of generator (Corn and Esmen, 1976). However, a 0.1% solution of urethane yielded much smaller particles, again due to sublimation. The MMD for 0.1%, 1% and 10% solutions of urethane were 0.02, 0.07 and 0.08 resp., and particles of such sizes will easily reach the alveolar region in small animals (Palm et al., 1956).

Thus, the airborne concentrations of urethane reported from calibration of the generator output includes solid particles of urethane as well as its vapor. This makes it difficult to estimate pulmonary retention on the basis of particle size (Palm et al., 1956) to calculate the dose received (see below) but we assumed the maximum possible, i.e. 100%, as a guide for the intravenous infusion and intraperitoneal injection.

Infusion and injection of urethane

The actual dose of urethane for each mouse during inhalation at 0.90 mg/l (10% solution of urethane) was estimated by the following equation:

$$D = \frac{C \times MV \times t \times \alpha}{\text{b.w.}}$$

where

C = Concentration of urethane in air, 0.90 mg/l
MV = Minute volume = $RR \times TV$
RR = Respiratory rate (250 respirations/min)
TV = Tidal volume (0.1 ml)
t = Duration of exposure in min, i.e. 240
α = Per cent retention estimated at 100%
b.w. = body weight in kg.

D was found to be 193 mg/kg. For intravenous infusions of an equivalent D, urethane solutions of 122 mg/10 ml concentration were infused at a flow rate of 0.125 ml/h for 4 h, 11:15 a.m.—3:15 p.m. A recently described procedure was used for infusion (Conner et al., 1980b). Groups consisting of 2 hepatectomized and 2 intact mice were treated at the same time. Intravenous treatment was repeated to obtain 4 mice of each kind. Non-treated controls were included with each group. For intraperitoneal injections, a single dose amounting to 193 mg/kg was given just prior to incorporation of 5-bromo-2′-deoxyuridine (BrdU).

BrdU incorporation

Immediately following urethane exposure by the previously described routes , mice were given continuous intravenous infusions of BrdU solutions (in PBS 13 mg/ml) at a flow rate of 3.0 ml/24 h. Using a simplified infusion device (Conner et al., 1980b), mice were infused for 17 h during which they had access to food and water ad libitum. At 9:00 a.m. corresponding to day 6 post-hepatectomy, mice were given an intraperitoneal injection of 100 μg of colchicine.

Cell harvest, staining and scoring

Mice were sacrified by cervical dislocation at 1:00 p.m. Cell harvest and staining procedures were as previously described (Conner et al., 1979a, 1978). Slides were coded and blindly scored. For each cell type from each animal, 25 second division metaphases were scored for SCE and 100 consecutive metaphases were examined for numbers of first and second division cells.

Results

The effect of increasing urethane exposure concentration is summarized in Fig. 1 and Table 1. Clear dose—response relationships were apparent in all cell types examined in hepatectomized and intact mice. In all cell types, no significant increases in SCE frequencies were observed relative to corresponding controls at 0.035 mg/l. However, at concentrations of 0.1 mg/l and higher, induced SCE frequencies were linearly related to log urethane concentrations as described by the linear regression equations in Fig. 1. Testing of slopes (Finney, 1971) revealed no significant differences in SCE response between like cell types of hepatectomized and intact mice. In hepatectomized mice, there was no significant difference in the SCE response of regenerating liver and alveolar macrophage cells. However, bone-marrow response was significantly lower (p = 0.01). Likewise, in intact mice bone-marrow response was significantly lower than that of alveolar macrophages (p = 0.01).

It is also apparent from Table 1 that inhalation and intravenous infusions of the same total dose of urethane (193 mg/kg) administered over a 4-h period produced comparable SCE responses in all cell types. However, a single intraperitoneal injection of 193 mg/kg just prior to BrdU infusion produced significantly higher SCE frequencies in bone marrow (α = 0.01), and alveolar macrophages (α = 0.05) of intact mice than did the equivalent inhalation dose. Intraperitoneal injections produced similar results in 2-month-old mice as in 4-month-old mice. However, regardless of the route of administration SCE frequencies in regenerating liver and/or alveolar macrophages were significantly higher than in bone marrow.

Discussion

In the present study a single 4-h exposure to aerosolized urethane was found to be 10—100 times more effective than previously found for styrene (Conner et al., 1980a) in inducing SCE in bone marrow, alveolar macrophages, and regenerating liver cells of hepatectomized mice and in bone marrow and alveo-

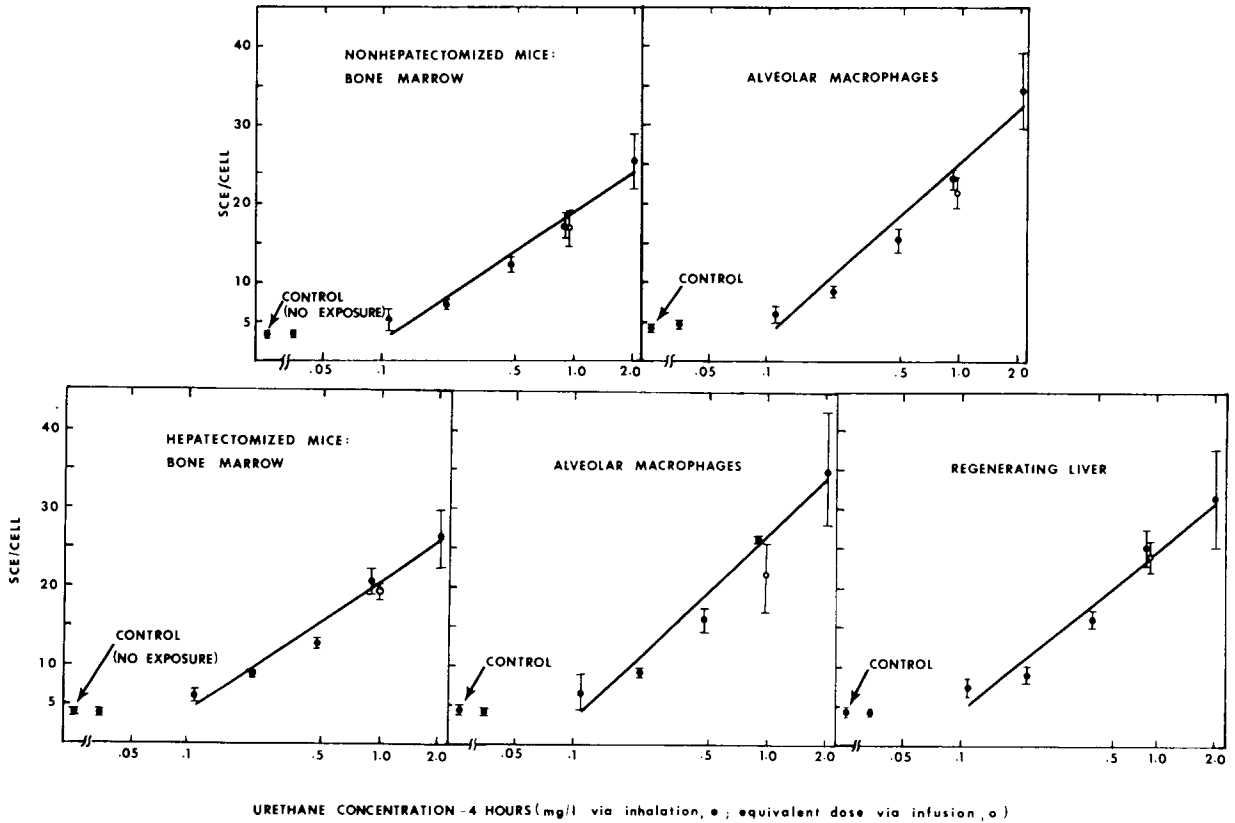

Fig. 1. Effect of inhalation of urethane on Mean frequencies of SCE per cell in hepatectomized and intact mice. The data points represent the mean ± S.D. as given in Table 1. Linear regression equations of the form, SCE/cell = $b + m$ log urethane concentrations:

		b	m	Correlation coefficient
Intact mice	Bone marrow	18.8	15.9	0.98
	Alveolar macrophages	25.2	22.7	0.98
Hepatectomized mice	Bone marrow	20.6	16.6	0.98
	Alveolar macrophages	26.3	23.2	0.98
	Regenerating liver	24.8	20.4	0.98

TABLE 1

EFFECT OF URETHANE TREATMENT ON PERCENTAGE OF SECOND DIVISION CELLS AND SISTER-CHROMATID EXCHANGE (SCE) FREQUENCIES IN BONE MARROW, ALVEOLAR MACROPHAGE, AND REGENERATING LIVER CELLS

Mean SCE per cell ±S.D. (column A) and percentage of second division cells per 100 consecutive metaphases (column B).

Exposure route	Urethane concentration	Bone marrow		Alveolar macrophage		Regenerating liver	
		A	B	A	B	A	B
Hepatectomized mice							
No exposure	0	3.8 ± 0.4	75	3.9 ± 0.5	63	4.3 ± 0.7	65
Inhalation							
(mg/l, 4 h)	0.035	3.9 ± 0.4	81	4.1 ± 0.3	70	4.3 ± 0.2	81
	0.11	6.1 ± 0.7	70	6.8 ± 2.3 [a]	61	7.6 ± 1.0 [a]	66
	0.22	8.9 ± 0.4	70	9.2 ± 0.5	76	9.0 ± 1.0	80
	0.48	12.9 ± 0.3	66	15.9 ± 1.6	75	16.2 ± 1.0	79
	0.90 [c]	20.6 ± 1.5	77	26.5 ± 0.3	62	25.5 ± 2.2	67
	2.06	26.0 ± 4.1	52	34.8 ± 7.7	41	31.7 ± 6.3	50
Intravenous infusion							
(mg/kg/4 h) [c]	193 mg/kg [c]	19.4 ± 0.7	68	21.7 ± 4.7	69	24.1 ± 1.9 [b]	58
Non-hepatectomized mice							
No exposure	0	3.7 ± 0.2	72	4.3 ± 0.5	41		
Inhalation							
(mg/l, 4 h)	0.035	3.6 ± 0.2	80	4.8 ± 0.4	51		
	0.11	5.2 ± 1.1	67	6.0 ± 1.0	39		
	0.22	7.3 ± 0.4	78	8.9 ± 0.4	50		
	0.48	12.2 ± 0.7	80	15.6 ± 1.3	72		
	0.90 [c]	17.1 ± 1.5	74	23.3 ± 1.3	57		
	2.06	25.3 ± 3.2	46	34.5 ± 4.8	33		
Intravenous infusion							
(mg/kg/4 h) [c]	193 [c]	16.8 ± 2.3 [a]	80	21.4 ± 1.0 [a,b]	54		
Intraperitoneal							
injection (mg/kg)	193 [c]	21.6 ± 0.9 [a]	64	26.3 ± 2.9 [a]	36		
	193 [d]	23.2 ± 0.2	48	29.7 ± 2.5	47		
	0 [d]	3.8 ± 0.1	66	4.6 ± 0.2	50		

All exposed groups had 4 mice in each category. All non-exposed controls had 8 mice in each category. 25 metaphases were scored from each animal in each category.

[a] Mean is based on 3 animals.

[b] 1 hepatectomized mouse has only 19 liver metaphases available for scoring. 1 intact mouse had only 19 alveolar macrophages available for scoring.

[c] Total doses administered were equivalent at 193 mg/kg/4 h.

[d] Mice were 2 months old.

lar macrophage cells of intact mice. Comparison of urethane concentration—SCE response relationships revealed increased tissue susceptibilities in regenerating liver and/or macrophage cells relative to bone marrow. Similar relative specificities were observed following urethane administration by inhalation or intravenous infusion. Comparable relative tissue SCE sensitivities were recently reported for hepatectomized mice wherein regenerating liver cells demonstrated greater sensitivity than did bone marrow following intraperitoneal injections of urethane (Roberts and Allen, 1980). The additional observation concerning alveolar macrophage SCE response in the present study parallels tissue suscepti-

bilities for urethane-induced tumors wherein alveolar adenomas resulted following various routes of administration (Shimkin and Stoner, 1975). Furthermore, in hepatectomized mice, comparable SCE responses observed in regenerating liver and alveolar macrophage cells are consistent with the previously reported similarity in binding of urethane to DNA in liver and lung cells (Bhide, 1974) as well as the increased susceptibility of male mice to hepatomas (Chernozemski and Warwick, 1970; Hollander and Bentvelzen, 1968) following partial hepatectomy.

The congruity in dose—SCE effect relationships demonstrated by like cell types in hepatectomized and intact mice suggests lack of an effect due to expected impaired liver function in hepatectomized mice. It is interesting to note that hepatectomy also failed to alter the incidence of alveolar adenomas over that found in intact mice (Nomura, 1976). Moreover, in the present study hepatectomy failed to alter the cytotoxicity of urethane as indicated by lack of reduction in the percentage of second division metaphase cells relative to that of controls. Similar consistent decreases in second division metaphases were observed in hepatectomized and intact mice only at the highest aerosol concentration employed (Table 1). These results are consistent with the reported metabolic capability of lung wherein the action of inducers indicated that lung is able to alter urethane in situ to an active metabolite, hydroxyurethane (Yamamoto et al., 1971) which reportedly is more effectively eliminated from liver than lung of intact mice (Nery, 1968).

In other studies, urethane was not found to be an effective SCE inducer in Chinese hamster cells in vitro (Abe and Sasaki, 1977). However, SCE induction by urethane and hydroxyurethane occurred in metabolically capable human lymphocytes cultured in vitro, but this effect was diminished by prior treatment with liver microsomal fractions (Csukas et al., 1979). Although the role of urethane and/or hydroxyurethane in SCE induction remains to be elucidated, the potency demonstrated in our studies further attests to the value of an in vivo assay as an indicator of biological effects in an animal.

No age effect was apparent between 2- and 4-month-old intact mice receiving intraperitoneal injections of urethane, but this route of administration generally produced higher SCE frequencies than did a comparable total dose administered via inhalation or intravenous infusion. This difference may be the result of a higher peak acute dose produced following the single intraperitoneal injection as contrasted with the same total dose given over a 4-h period of infusion or inhalation.

The relationship between urethane-induced neoplasia and specific cellular hyperplastic responses remains to be elucidated. However, several studies have described urethane-induced preneoplastic hyperplasia characterized by a general increase in mitotic activities of alveolar type-I and type-II epithelial cells as well as alveolar macrophages (Dyston and Heppleston, 1976; Kauffman, 1974). Since alveolar wall hyperplasia is presumably initiated by cellular damage (Kauffman, 1971), alveolar macrophages and their monocyte precursors, by virtue of their interstitial residence, are susceptible to cytotoxic effects of active chemicals in the alveolar environment. The cellular SCE specificities for alveolar macrophages and regenerating liver cells observed in the present study, which parallel tissue specificities previously reported for urethane binding and

tumorogenesis, now makes it possible to evaluate the relationship of specific tissue SCE responses to mutagenesis and carcinogenesis following administration of urethane in vivo.

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