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Short communication

Urethane and *N*-nitrosodiethylamine are mutagenic for the Syrian hamster fetus

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ABSTRACT

Urethane and *N*-nitrosodiethylamine are soluble environmental carcinogens that initiate tumors transplacentally, but have a mixed history of effectiveness in mutagenesis assays *in vitro* or *in vivo* with adult rodents. To test for their transplacental mutagenicity, Syrian hamster fetuses at 12 days in gestation were exposed transplacentally to urethane or *N*-nitrosodiethylamine at 0.5 or 1.0 mM/kg. The fetal cells were isolated on day 13 of gestation and tested for diphtheria toxin resistance as a mutation marker. Both compounds were significantly mutagenic, at both doses, causing 6- to 20-fold increases in mutations compared with controls. Compared with *N*-nitrosodiethylamine, urethane was somewhat more effective as a mutagen with a more marked dose–response. These results are consistent with mutagenesis as part of the mechanism of transplacental carcinogenicity of urethane and *N*-nitrosodiethylamine.

1. Introduction

Urethane [1] and *N*-nitrosodiethylamine (NDEA) [2] are low molecular weight environmental chemical carcinogens, which are active transplacentally. Both are thought to be metabolically activated mainly or in part by cytochrome P450 2E1 [3]. Urethane may be oxidized by CYP2E1 to vinyl carbamate which then yields a reactive epoxide [4]. By use of knockout mice Hoffler et al. [5] demonstrated that cyp2E1 is the principle enzyme responsible for urethane metabolism to CO_2. Loss of cyp2E1 in these mice also reduced urethane-induced genotoxicity and cell proliferation [6]. Several types of evidence indicate that NDEA is metabolized by CYP2E1 and 2A6 to reactive intermediates [7]. The first step was suggested to be hydroxylation of the carbon atom at the α-position of the nitroso group [8].

It is presumed that the activated forms of both chemicals cause transformation as a result of DNA damage. NDEA causes DNA strand breaks, oxidative base damage, DNA–protein cross-links, chromosomal aberrations, and pro-mutagenic alkylation products at the O^6 position of deoxyguanosine and O^4 of deoxythymidine [9–12]. Urethane is clastogenic [13] and leads to 7-[2′-oxyethyl]deoxyguanosine adduct via its metabolite vinyl carbamate [14,15]. 1,N^6-ethenodeoxyguanosine and 3,N^4-ethenodeoxycytidine have also been demonstrated [16,17], and the urethane metabolite *N*-hydroxyurethane caused oxidative DNA damage [18].

However, findings with mutagenesis assays have been mixed. Urethane was negative in the Ames assay [19], but positive in the *in vivo* mouse micronucleus test [20], *in vivo* sister chromatid exchange (SCE) assay [21], and the *in vitro* chromosome aberration test [22]. It was also mutagenic in *Drosophila* [23,24]. NDEA was negative in the regular Ames assay test, in the *in vivo* mouse micronucleus test [20], in the mouse spot test [25], and the mouse specific loci test [26], but positive using the Ames liquid incubation assay [19], in the *in vitro* hepatocyte unscheduled DNA synthesis (UDS) assay [27], and in the transplacental micronucleus test [28].

Pregnant mice have a changing response to urethane at different times of gestation [29,30]. With exposure to urethane before implantation on day 3, pre-implantation loss was observed. Exposure on day 7 caused complete resorption of the embryo. Early deaths were observed only after exposure on day 8 and late deaths after exposure on days 8–11. Malformations occurred in the lung, at a maximum when exposure was on day 9 and liver abnormalities peaked after treatment on day 8. Tumors were induced in the liver when exposure to urethane occurred from day 11 to 17 (1000 mg/kg) or day 15 to 17 (200 mg/kg). Lung tumors were numerous after urethane treatment from day 13 to birth, with absolute numbers peaking after exposure on day 15.

Transplacental NDEA was embryotoxic in the rat after treatment on days 3, 9, 10, and 12 [31]. Treatment of pregnant mice with NDEA (45 mg/kg) on different days of gestation yielded lung tumors which increased in number with day of gestation of treatment [32]. Day 15 treatment yielded no lung tumors; exposure on days 16, 17 and 18 yielded 24%, 73% and 87% respectively. NDEA given to pregnant hamsters on different days of gestation initiated tumors only on days 12–15 [2]. These were mostly respiratory tract tumors.

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Table 1
Effects of ENU, urethane, and NDEA on cloning efficiency and mutation rate and frequency in fetal hamster cells

Treatment dose as mmol/kg	Cloning efficiency (%) ± SE	Mutants per plate ± S.E.	Mutants per surviving cell × 10^{-7} ± S.E.
Control	53.5 ± 1.6	0.2 ± 0.1	3.740 ± 1.72
ENU 1.0	31.4 ± 0.7[a]	4.16 ± 0.56[a]	132.57 ± 18.02[a]
Urethane 0.5	50.2 ± 3.3	1.76 ± 0.33[b]	35.15 ± 6.51[c,f]
Urethane 1.0	28.7 ± 3.8[a]	2.15 ± 0.37[a]	73.40 ± 12.43[a,f,g]
NDEA 0.5	60.1 ± 1.8[d]	1.42 ± 0.38[e]	23.64 ± 6.40[e]
NDEA 1.0	46.1 ± 1.9 [d]	1.55 ± 0.28[c]	33.62 ± 5.99[b,g]

Mutants were selected with diphtheria toxin.
[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$, significance of difference as compared with control in ANOVA or Kruskal–Wallace test.
[d] Significantly different, $P < 0.001$, ANOVA.
[e] Significantly different from controls, $P < 0.01$, pairwise test.
[f] Significantly different, $P = 0.01$, unpaired t test with Welch correction.
[g] Significantly different, $P = 0.0076$, unpaired t test with Welch correction.

It was therefore of interest to determine whether the transplacental carcinogenic effects of these chemicals involve mutagenesis. We have treated pregnant Syrian hamsters on gestation day 12 with urethane or NDEA and isolated fetal cells on day 13 for measurement of mutations. Both compounds proved to be transplacental fetal mutagens.

2. Materials and methods

Our report of a previous study [33] has outlined in detail the materials and methods used. Briefly, pregnant Syrian hamsters were obtained from the Animal Production Area of the Frederick Cancer Research and Development Center, Frederick, MD. On day 12 of gestation the pregnant animals were administered i.p. freshly prepared urethane (Eastman Kodak Co.) or NDEA (Eastman Kodak Co.) in sterile phosphate buffered saline (Biofluids) at the doses indicated. Ethylnitrosourea (ENU, 1 mmol/kg) was administered as a positive control. ENU was synthesized by John Klose, SAIC, NCI-Frederick [34]. Animals were exposed using the procedures as outlined [33]. On day 13 of gestation fetuses were isolated and minced and then trypsinized. The pooled trypsinate from each litter was frozen at 1° min^{-1} and stored above liquid nitrogen. Cells were thawed and cultured *in vitro* for a 5-day expression period. We had previously demonstrated that with this dose of ENU, the mutant frequency was constant at 3–6 days expression time and decreased thereafter (data not shown). No cytotoxic cell loss was noted during the expression period. After the expression time we seeded at 300 cells/100 mm plate (5 plates/litter) for cloning efficiency or 1×10^6 cells/100 mm plate for selection by diphtheria toxin (DT) (20 plates/litter). Because Syrian hamster cells historically had low cloning efficiency we used a rat cell feeder layer. For both cloning efficiency and selection plates, this feeder layer consisted of a 24 h culture of 1.6×10^6 X-rayed rat embryo fibroblast cells/100 mm plate. As rat cells are resistant to DT we could use this chemical as a selective agent. Cloning efficiency plates were fixed and stained 7 days after seeding while selection plates were fixed and stained 3 weeks after seeding.

3. Results

In a previous study [33] the spontaneous mutant frequency was determined for 26 hamster litters. We found 77 mutants in 840 plates (0.09 mutants/plate) to give an overall historical mean of 2.6×10^{-7} mutants/surviving cell, with an upper 95% tolerance limit of 7.3×10^{-7}. In the present study the untreated control litter had a spontaneous mutant frequency per surviving cell of 3.7×10^{-7} (Table 1), within the 95% confidence interval for our historical control cells [33]. The positive control, ENU at 1 mM/kg, caused a significant reduction in cloning efficiency, and was highly mutagenic as expected, with 21- and 36-fold increases in mutants/plate and mutants/surviving cell, respectively.

Urethane caused a significant reduction in cloning efficiency only at the higher, 1.0 mM/kg dose. Both doses caused significant, 9- to 20-fold increases in mutants per plate and mutants per surviving cell, compared with concurrent controls (Table 1). With the concurrent control values included, a significant dose–response was demonstrated for urethane-caused mutants per surviving cell ($P = 0.036$, $r = 0.998$ in linear regression analysis). Relative to historical control values determined with a large number of litters (see above), 35.2 and 73.4×10^{-7} mutants per surviving cell for the two urethane doses were 5- and 10-fold greater than the upper 95% tolerance limit. This confirms their significance.

There was a small but significant difference between the two NDEA doses with regard to cloning efficiency, though neither was significantly different from controls. Both doses resulted in significant 6- to 9-fold increases in mutants per plate and per surviving cell, compared with concurrent controls. Compared with historical controls, 23.6 and 33.6×10^{-7} mutants per surviving cell were confirmed as significant as they were more that 2-fold greater than the upper 95% tolerance limit for the control cells. There was possibly greater effectiveness of the higher dose with regard to mutants per surviving cell, although short of statistical significance ($P = 0.12$, $r = 0.982$ in linear regression analysis). In a pairwise statistical test, NDEA at 1.0 mM/kg was less mutagenic per surviving cell than was urethane at the same dose.

4. Discussion

In spite of negative results in several mutagenesis assays, in the current study both urethane and NDEA were unequivocally mutagenic to cells of the developing Syrian hamster fetus near the end of gestation. Urethane was the more toxic, demonstrated a clearer dose–response, and was somewhat more mutagenic than NDEA at the higher dose of 1 mM/kg. These differences are consistent with the known toxic effects of urethane throughout gestation in the mouse, with lung, liver, and ovarian tumors resulting from exposures at gestation days 11–13 [30]. Transplacental urethane caused sister chromatid exchanges in fetal mouse tissues [35]. Radioactive urethane given subcutaneously to pregnant mice was present in equal concentrations in organ tissues and body fluids of both pregnant mice and their fetuses at all stages of gestation and was catabolized rapidly within 6 h [29]. Tumors initiated in fetal lung, relative to organ weight, decreased in number as gestation progressed, suggesting that proliferation rate rather than tissue differentiation determined sensitivity [29]. A similar differential was observed for sister chromatid exchanges caused in fetal liver by urethane [35]. It is possible that activated but somewhat stable metabolic intermediates are formed by maternal enzymes, especially cyp2E1 in the liver, with subsequent delivery to the fetus. This is suggested by the fact that urethane was only slowly metabolized in newborn mice [36].

Metabolism of NDEA, on the other hand, occurs in hamster fetal tissues only toward the end of gestation in correlation with appearance of the endoplasmic reticulum [37–39]. Developmental appearance of metabolic capability was associated with occur-