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In Vitro and *In Vivo* Genotoxicity of 1,3-Butadiene and Metabolites

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1,3-Butadiene and two major genotoxic metabolites 3,4-epoxybutene (EB) and 1,2:3,4-diepoxybutane (DEB) were used as model compounds to determine if genetic toxicity findings in animal and human cells can aid in extrapolating animal toxicity data to man. Sister chromatid exchange (SCE) and micronucleus induction results indicated 1,3-butadiene was genotoxic in the bone marrow of the mouse but not the rat. This paralleled the chronic bioassays which showed mice to be more susceptible than rats to 1,3-butadiene carcinogenicity. However, 1,3-butadiene did not induce unscheduled DNA synthesis (UDS) in the rat or mouse hepatocytes following *in vivo* exposure. Likewise, UDS in rat and mouse hepatocytes *in vitro* was not induced by EB or DEB. *Salmonella typhimurium* gene mutation (Ames) tests of 1,3-butadiene using strains TA1535, TA97, TA98, and TA100 and employing rat, mouse, and human liver S9 metabolic systems were barely 2-fold above background only in strain TA1535 at 30% 1,3-butadiene in air with induced and uninduced rat S9 and mouse S9 (uninduced). 1,3-Butadiene was negative in *in vitro* SCE studies in human whole blood lymphocytes cultures treated in the presence of rat, mouse, or human liver S9 metabolic activation. In general, 1,3-butadiene is genotoxic *in vivo* but is a weak *in vitro* genotoxin.

Introduction

1,3-Butadiene is a potent carcinogen in B6C3F$_1$ mice *(1,2)* but only weakly tumorigenic in Sprague-Dawley rats *(3)*. These differences in carcinogenic potency have raised the question as to which of the two species, if either, is the better indicator of the human response. Studies evaluating the mutagenic response to 1,3-butadiene *in vivo* and *in vitro* in rodents and humans should permit a more quantitative estimation of toxicological differences between man and rodents. We examined the possibility that the genotoxicity of 1,3-butadiene or genotoxic metabolites is a major contributing factor to its carcinogenic activity in rodents.

Two known genotoxic metabolites, 3,4-epoxybutene (EB) and 1,2:3,4-epoxybutane (DEB) are formed *in vivo (4)* and *in vitro (5,6)* and both bind covalently to DNA *(7–9)*. 3,4-Epoxybutene was reported to be genotoxic in Klebsiella and Salmonella *(10)* and induce sister chromatid exchange and chromosome aberrations in mice *(11,12)*. DEB was also genotoxic in a variety of prokaryotic and eukaryotic organisms *in vitro (13–17)*.

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1,3-Butadiene has been shown to be genotoxic in the Salmonella mutation assay *(18–20)* and *in vivo* in mice *(21–23)*; it is, however, negative in rats *(21)*. Since 1,3-butadiene must be metabolized to exhibit genotoxicity *(19)*, the metabolic competence of different species may determine the putative hazard in that species. Preliminary findings indicated that mouse liver homogenates are more active in producing EB than corresponding rat or primate (including human) homogenates *(24)*. Similarly, mouse lung homogenates generate more metabolites than rat lung homogenates. Kreiling et al. *(25)* reported that the maximal metabolic elimination rate of 1,3-butadiene in the mouse is nearly twice that of the rat. Blood concentrations of EB were two to five times higher in 1,3-butadiene-treated B6C3F$_1$ mice than Sprague-Dawley rats *(4)*. We chose to look at the effects of different species sources of liver homogenates as our activation system to determine if 1,3-butadiene metabolism was a contributing factor to the genotoxic differences between species.

Salmonella typhimurium Assay Using Rat, Mouse, and Human S9

Studies by de Meester et al. *(19)* indicated that 1,3-butadiene induced mutations in *Salmonella typhimurium* TA1530 in the presence of Aroclor 1254-induced rat liver S9 and mouse liver microsomes. No mutations in the presence of uninduced rat liver S9 were observed. We

Table 1. Revertants of strain TA1535 treated with 1,3-butadiene with and without various S9 activations systems.[a]

% 1,3-butadiene (in air)	TA1535 revertants/plate				
	Rat S9 Aroclor	Rat S9 uninduced	Mouse S9 uninduced	Human S9 uninduced	No S9
0	21 ± 4	20 ± 0	32 ± 4	22 ± 10	25 ± 4
30	55 ± 5	42 ± 5	68 ± 6	32 ± 12	31 ± 0
40	48 ± 1	NT[b]	50 ± 10	41 ± 1	37 ± 2
50	52 ± 15	41 ± 4	47 ± 1	39 ± 3	43 ± 1
60	40 ± 6	NT	31 ± 3	42 ± 6	37 ± 4
2-AA[b]	1237 ± 7	200 ± 52	255 ± 14	578 ± 13	
NaAz[b]					888 ± 2

[a]All S9 concentrations were 0.8 mg protein/plate. Experimental error is expressed as standard deviation between plates.
[b]Abbreviations: NT, not tested; 2-AA, 2-aminoanthracene, 2 μg/plate; NaAz, sodium azide, 2 μg/plate.

investigated the capability of Aroclor 1254-induced rat, and uninduced rat, mouse, and human S9 to activate 1,3-butadiene into genotoxic metabolites as detected by the Salmonella mutation assay *(26)* using strains TA1535, TA97, TA98, and TA100. All S9 preparations were made according to the procedure of Ames et al. *(26)*. Design concentrations of 1,3-butadiene gas were metered into specially constructed treatment chambers holding the agar plates overlaid with the bacteria and activation system. Actual gas concentrations were determined by gas chromatographic analysis before and after the 48-hr exposure. Because 1,3-butadiene is explosive at concentrations between 2 to 12% in air, the lowest dose that could be tested safely was 30%. Different treatment chambers were used for each activation system and for the nonactivated treatment.

1,3-Butadiene induced revertants only in strain TA1535 (Table 1). Mouse S9 showed slightly higher activity than the uninduced rat or human S9 at 30% 1,3-butadiene in air. At concentrations exceeding 30%, the number of revertants decreased in the presence of rat or mouse S9. Results from the human S9-activated treatments did not differ substantially from those of the nonactivated treatments. Aroclor 1254-induced rat S9 gave similar results as mouse S9 (uninduced). Since the response was weak, the S9 concentration was increased from 0.8 mg/plate to 4.0 mg/plate (Table 2). Increasing the concentration of Aroclor 1254-induced rat S9 had no effect on the number of revertants; slightly more revertants were observed using 4.0 than 0.8 mg/plate of uninduced rat S9.

These *Salmonella typhimurium* results differ from de Meester's *(19)* in two ways. First, the level of mutagenic activity was substantially lower with induced rat S9 than reported by de Meester *(19)*. Second, we were able to detect a weak response in strain TA1535 with uninduced rat S9. Differences in 1,3-butadiene and S9 protein concentrations and strains TA1530 versus TA1535 are probably the source of this interlaboratory variation.

Table 2. Revertants of strain TA1535 treated with 1,3-butadiene with varying concentrations of Aroclor 1254-induced and uninduced rat S9.

% 1,3-butadiene (in air)	Uninduced rat S9		Aroclor® 1254-induced rat S9	
	0.8 mg/plate	4.0 mg/plate	0.8 mg/plate	4.0 mg/plate
0	20 ± 0	21 ± 3	23 ± 2	18 ± 1
30	42 ± 5	60 ± 10	45 ± 3	46 ± 1
50	41 ± 4	57 ± 3	48 ± 2	50 ± 24
2-AA[a]	200 ± 52	NT[a]	TNTC[a]	NT[a]

[a]Abbreviations: 2-AA, 2-aminoanthracene, 2 μg/plate; NT, not tested; TNTC, too numerous to count.

Human Whole Blood Lymphocyte SCE Assays

Human whole blood lymphocytes were also used to investigate the ability of rat, mouse, and human S9 to activate 1,3-butadiene to genotoxic metabolites using SCE as the biological marker *(27)*. Design concentrations of 1,3-butadiene gas were metered into specially constructed treatment chambers holding the culture flasks. Actual gas concentrations were determined by gas chromatographic analysis.

The average generation time and mitotic index of the cultured cells were unaffected by 1,3-butadiene treatment in the presence of S9; although in the presence of 1,3-butadiene and mouse S9, cells were beginning to demonstrate a dose-responsive decrease in average generation time (data not shown).

No positive SCE or dose responses were observed with or without any of the activation systems at 25 to 100% 1,3-butadiene in nitrogen (Table 3). In contrast,

Table 3. Sister chromatid exchanges in human whole blood lymphocytes treated with 1,3-butadiene in the absence and presence of rat, mouse, or human S9.[a]

% 1,3-butadiene (in N_2)	Rat S9 Aroclor	Rat S9 uninduced	Mouse S9 uninduced	Human S9 uninduced	No S9
0	4.3 ± 1.4	4.9 ± 1.1	8.4 ± 2.6	4.6 ± 1.6	3.8 ± 0.8
25	3.8 ± 0.7	5.9 ± 1.1	7.0 ± 2.0	5.1 ± 1.0	4.1 ± 0.7
100	4.3 ± 0.5	6.2 ± 0.3	9.3 ± 2.3	5.1 ± 0.7	4.9 ± 0.8
CP[b]	CA[b]	11.1 ± 2.1	20.8 ± 8.2	7.5 ± 2.5	
MMC[b]					19.7 ± 0.8

[a]Male and female human lymphocytes were used for each study with a different preparation of liver S9 fraction used for the second trial. Only one trial was performed with Aroclor 1254-induced rat S9. Whole blood cultures were initiated by inoculating 0.3 mL of whole blood into 4.7 mL of supplemented RPMI 1640 with 1.5 to 1.9% reconstituted phytohemagglutinin. Cultures were incubated 48 hr prior to treatment. All activated treatments contained S9 at a final concentration of 1 mg protein/mL. Cultures were exposed to 1,3-butadiene for 4 hr, washed; 5 mL culture medium contained 10 μM BrdU was added; 2 hr prior to a 24-hr harvest 0.1 μg/mL colcemid was added. SCE analysis was performed as described by Cunningham et al. *(21)*. At least 25 cells per treatment group were scored. Experimental error is expressed as the standard deviation between cultures.
[b]Abbreviations: CP, cyclophosphamid, 25 μg/mL; MMC, mitomycin C, 0.25 μg/mL; CA, chromosomal aberrations were observed in cells in first division; however, toxicity was too excessive to score SCEs which required cells to have completed two rounds of cell division.

Table 4. *In vivo/in vitro* unscheduled DNA synthesis in rat and mouse hepatocytes in animals exposed to 10,000 ppm 1,3-butadiene.[a]

Treatment[b]	Net grain count cell	
	Mouse	Rat
Air, 2 hr	−0.9 ± 0.3	−1.7 ± 0.4
1,3-Butadiene, 2 hr	+0.4 ± 0.4	−0.6 ± 0.6
DMBA	+16.0 ± 2.0	+45.2 ± 10.4
Air, 18 hr	−6.3 ± 1.2	−4.1 ± 0.8
1,3-Butadiene, 18 hr	−3.7 ± 0.6	−5.2 ± 0.8
DMBA	+19.8 ± 6.3	+40.8 ± 4.4

[a]The air control and each 1,3-butadiene treatment consisted of two animals per species with two slides scored per animal. A minimum of 100 cells were scored for each treatment group. The positive control was treated *in vitro* from the animals used in the air exposure. A minimum of 75 cells were scored. The error is expressed as the SE of the mean between the average net grain counts per slide in each treatment.

[b]Abbreviations: 1,3-butadiene, 2 hr = 1,3-butadiene exposure for 6 hr on day 1 and 3 hr on day 2 with 2 hr postexposure time (repair time); 1,3-butadiene, 18 hr = 1,3-butadiene exposure for 6 hr for 2 days with a 18-hr postexposure time; DMBA, dimethylbenzanthracene (100 μM) 18-hr treatment *in vitro*.

both cyclophosmid and mitomycin C induced SCEs for activated and nonactivated treatments, respectively.

In Vivo/In Vitro Unscheduled DNA Synthesis and *In Vitro* Assays

In the *in vivo/in vitro* unscheduled DNA synthesis (UDS) assay *(28)*, two 1,3-butadiene exposure/sampling protocols were employed. Male Sprague-Dawley (Crl:CD BR) rats and B6C3F$_1$ (B6C3F$_1$/CrlBR) mice were exposed nose-only to either air or air containing 10,000 ppm 1,3-butadiene for *a)* 6 hr on day 1 followed by 3 hr on day 2; livers sampled 2 hr after the end of the second exposure, and *b)* 6 hr/day for 2 days; livers sampled 18 hr after the second exposure. [See Cunningham et al. *(21)* for inhalation conditions.] No UDS was evident in either species under those conditions (Table 4).

In the UDS assay in rodent hepatocytes *in vitro (29)*, test concentrations of EB and DEB ranged from 5 to 1000 ppm in medium. Both metabolites were cytotoxic above 5 ppm, as evidenced by a decrease in nuclear and cytoplasmic grain counts. No cell survival was obtained at concentrations above 1000 ppm. Both EB and DEB failed to induce UDS in rat and mouse hepatocytes. Slight increases in the net nuclear grain counts were evident; however, they appear to be the result of DEB- and EB-induced cytotoxicity, as evidenced by a significant drop in grain counts over the cytoplasm. Table 5 displays an example of the effect of EB on rat hepatocyte cultures.

In Vivo Genotoxicity in Rodent Bone Marrow

Male Sprague-Dawley rats and B6C3F$_1$ mice were exposed to 1,3-butadiene by nose-only inhalation at concentrations of 10 to 10,000 ppm for 6 hr/day for 2 days. SCE and micronucleus (MN) induction were subsequently measured in the bone marrow *(21)*. Toxicity of 1,3-butadiene was observed at 10,000 ppm as measured by polychromatic erythrocyte (PCE) suppression [expressed as the ratio of PCE/NCE (normochromatic erythrocytes)] in bone marrow. This was a possible indication that 1,3-butadiene and/or its metabolites reached the bone marrow and exerted toxicity.

Table 5. *In vitro* unscheduled DNA synthesis in rat hepatocyte cultures treated with 3,4-epoxybutene.[a]

3,4-Epoxybutene, ppm in medium	Nuclear grains/cell	Cytoplasmic grains/cell	Net grains/cell
0	22.4 ± 0.6	29.1 ± 0.8	−6.6 ± 0.7
5	27.2 ± 1.9	35.0 ± 1.9	−7.7 ± 2.0
50	11.1 ± 0.5	11.1 ± 0.2	+0.1 ± 0.5
100	9.3 ± 0.5	8.6 ± 0.1	+0.7 ± 0.5
1000	7.9 ± 0.5	7.1 ± 0.5	+0.8 ± 0.3
DMBA[b]	62.3 ± 4.8	33.4 ± 0.5	+29.0 ± 4.4

[a]3,4-Epoxybutene was dissolved in DMSO. Each treatment group consisted of four cultures with 25 cells scored per culture. The error is expressed as the SEM between cultures.

[b]DMBA, dimethylbenzanthracene.

Cunningham et al. *(21)* demonstrated MN frequency in the mouse bone marrow increased as a function of BD concentration, beginning at 100 ppm (0.44% micronucleated PCEs) and reaching 3.0% at 10,000 ppm. No increase above the control MN frequency (0.08%) was detected at or below 50 ppm. In comparison, there were no statistically significant increases in MN frequency in rats exposed to the same 1,3-butadiene levels as mice.

Analysis for SCE in the rat and mouse led to a similar conclusion. At 100 ppm, significant increases in SCE (20 SCEs/cell) were obtained in the mouse, reaching a maximal response of 30 SCEs/cell at 200 ppm compared to control levels of 8.5 SCEs/cell. The no effect level was 50 ppm. No significant effects were observed in the rat at concentrations up to 10,000 ppm *(21)*.

Discussion

1,3-Butadiene is genotoxic in the mouse but not in rat bone marrow cells. The genotoxic response in the mouse parallels the tumorigenicity data *(2)*. The results are also in agreement with the findings *(22,30)* that 1,3-butadiene affects mouse bone marrow stem cell development and induces macrocytic megaloblastic anemia. The absence of a genotoxic response in the rat also correlated relatively well with the low tumor incidence and absence of chronic toxicity in the hematopoietic system *(3)*. Because 1,3-butadiene failed to induce UDS in rodent hepatocytes *in vivo* nor did EB/DEB *in vitro*, it could be concluded that the genetic damage induced by BD or EB/DEB is not repaired by excision repair.

The differences in SCE and MN induction between the rat and mouse may be due to the metabolic and pharmacokinetic properties of 1,3-butadiene in the two

species. Schmidt and Loeser (*24*) demonstrated that mouse liver and lung postmitochondrial fractions, when treated with 30,000 ppm 1,3-butadiene, generated more EB than the respective rat homogenates. This argument, however, is weakened by the lack of a genotoxic response in human lymphocytes when using either Aroclor 1254-induced rat, uninduced rat, mouse, or human liver homogenates, and by the weak response of 1,3-butadiene in the Salmonella assay.

Our studies and those of others show that 1,3-butadiene is a potent *in vivo* genotoxin in the mouse, but a weak genotoxin *in vitro*. Physiological factors in the mouse not present in the *in vitro* test systems may contribute to the genotoxic effects in mouse bone marrow cells. The significance of these results to human risk is presently unknown.

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