Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 2148–2152, April 1980
Genetics

Cytogenetic effects of inhaled benzene in murine bone marrow: Induction of sister chromatid exchanges, chromosomal aberrations, and cellular proliferation inhibition in DBA/2 mice

(genetic toxicology/pollutants/clastogenic)

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Communicated by Richard B. Setlow, December 26, 1979

ABSTRACT **Exposure of adult male and female DBA/2 mice to 3100 ppm benzene for 4 hr significantly increased the frequency of sister chromatid exchanges in bone marrow cells of both sexes, inhibited marrow cellular proliferation (but only in male mice), and did not significantly increase the frequency of chromosomal aberrations in either sex. Phenobarbital pretreatment synergistically interacted with benzene exposure to further increase sister chromatid exchanges in female mice, induce greater inhibition of cellular proliferation in male mice, and induce a significant level of chromatid-type chromosomal aberrations in both sexes. During the second day after exposure to benzene there was increased inhibition of cellular proliferation in male mice and both new DNA damage and persistence of old DNA damage in female mice. The differences in both the type and magnitude of the response of bone marrow cellular populations, as determined by different cytogenetic end points in male and female DBA/2 mice exposed to benzene or to phenobarbital and benzene, suggest not only that a metabolite of benzene is responsible for the observed effects, but that different metabolites may be involved in different end points.**

The extensive use of benzene in industry and local commerce has resulted in both a large degree of human exposure and a considerable potential for environmental contamination (1). Acute exposure to benzene produces a dose-dependent depression of the central nervous system, whereas chronic exposure can lead to pancytopenia or aplastic anemia and, in some cases, leukemia (1–4). Both cellular toxicity and carcinogenic potential have been suggested to be causally related to an agent's clastogenic activity (5–7). Therefore, these chronic effects may be due to the observed ability of benzene to cause chromosomal aberrations in bone marrow cells of occupationally exposed workers and experimentally exposed animals (1–4). Recently, cytogenetic techniques have been developed that permit the simultaneous assessment of both sister chromatid exchanges (SCE) and cellular proliferation kinetics *in vitro* or *in vivo* (8, 9). SCE induction has been suggested to correlate with an agent's mutagenic/carcinogenic potential (10–12), and perturbations in cellular proliferation kinetics should correlate with cytotoxic effects. Therefore, we examined the ability of benzene to induce SCE or chromosomal aberrations (or both) and to affect cellular proliferation kinetics in murine bone marrow.

Recent evidence suggests that a liver-mediated metabolite(s) of benzene, rather than benzene itself, may be primarily responsible for benzene's interference with erythrocyte and leukocyte production in the bone marrow (13, 14). Phenobarbital, a well-known inducer of liver metabolism (15), has been used to assess the effect of increasing hepatic xenobiotic metabolism on the health effects of benzene (1–4). The results of these studies have been largely contradictory: phenobarbital has been shown to ameliorate benzene's effects in some cases and to increase it in others (1–4). To further examine this possible relationship between increased hepatic metabolism and the health effects of benzene, we incorporated phenobarbital pretreatment into our experimental design.

This approach, based on the simultaneous assessment of three separate cytogenetic end points, should allow insight into the nature of the benzene-induced cellular events leading to bone marrow depression and leukemia. The use of inhalation exposure provides a system directly comparable to normal human experience with benzene.

MATERIALS AND METHODS

Eight- to ten-month-old male (30–35 g) and female (25–30 g) DBA/2 mice, obtained as weanlings from Jackson Laboratory, were used throughout the experiments. Virgin animals were kept at a population density no greater than one animal per 97 cm^2 on corn cob bedding (Bed O'Cobs, J. R. Nielsen and Sons, S. Windsor, CT), which was changed weekly. Food (Purina Rodent Laboratory Chow 5001) and water were provided ad libitum. Animals were maintained on a 12-hr light:dark cycle (7 to 7) at 25°C; relative humidity was held at 50%, with 20 air exchanges per hr. Some animals were pretreated with sodium phenobarbital (50 mg/kg) or with control saline injected intraperitoneally twice daily for 3 days prior to benzene exposure. Randomized groups of mice were exposed in five separate runs to benzene vapor (mean value = 3130 ± 170 ppm) for 4 hr (12,500 ± 680 ppm·hr), beginning at 0900 hr. Exposures were conducted in an isolation chamber that has been described elsewhere (16). Control animals were similarly treated, but without exposure to benzene. Benzene vapor was generated by bubbling filtered compressed air through liquid benzene and diluting the vapor effluent appropriately. Benzene concentration was measured at half-hour intervals by a gas chromatograph (Packard, model 417, equipped with a column of 10% silicone SE-30 on chromasorb W-HP), with an automatic sampling valve.

One hour after exposure to benzene, exposed and control mice were infused with bromodeoxyuridine (BrdUrd, 50 mg/kg per hr) for various time intervals (generally, 24 hr) as described (17). Two hours prior to the termination of the infusion, Colcemid (GIBCO) was injected intravenously at a concentration of 1 μg/10 g body weight. Animals were killed by cervical dislocation, femurs were removed, and the marrow was flushed out with phosphate-buffered saline (pH 7.2). The resulting material was treated with hypotonic KCl and fixative; flame-dried slides were prepared and stained with the Hoechst 33258 (American Hoechst)/black light/Giemsa (Harleco)

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Abbreviation: SCE, sister chromatid exchanges.

technique as described (17). For determination of chromosomal aberration frequencies, 50 metaphase cells (first generation after benzene exposure only) were examined per animal on randomized slides by previously described criteria (17). Five animals were examined for each treatment. Where necessary, BrdUrd infusions of short duration (10 or 18 hr) were run to obtain comparable proportions of first-generation to subsequent-generation metaphase cells in bone marrow preparations in all animals. For SCE studies, 25 second-generation metaphase cells were examined in each animal to determine the mean number of SCE per cell. In addition, in some animals 25 third-generation metaphase cells were examined for the number of SCE that had been induced in the first two replication cycles or in the third replication cycle. In these metaphase cells, SCE induced during the first two replication cycles appear as nonsymmetrical disruptions in the longitudinal staining pattern of chromosomes, whereas SCE induced in the third replication cycle appear as symmetrical exchanges in the longitudinal staining pattern (18). Statistically significant differences between groups of animals were determined by Student's t test at the $P < 0.01$ level of significance. Cellular proliferation kinetics were assessed in each animal by determining, in 100 metaphase cells, the proportion of cells that had replicated for one (I), two (II), or three (III) cycles in the presence of BrdUrd (19). Statistically significant differences between groups of animals were determined by χ^2 analysis with $P < 0.01$ level for significance.

RESULTS

Inhalation of benzene by DBA/2 mice induced a significant increase ($P < 0.01$) in the level of SCE in bone marrow cells compared to unexposed controls (Table 1). The magnitude of this response was not significantly different between males and females (89% and 82%, respectively). This increase in SCE frequency occurred at a level of benzene exposure that did not significantly increase the frequency of chromosomal aberrations (Table 1), suggesting that, as for many other DNA-damaging agents (10–12), SCE induction may be a more sensitive indicator of agent activity than aberration yields. Phenobarbital pretreatment in itself had no effect on SCE or chromosomal aberration frequency. However, phenobarbital pretreatment did synergistically interact with benzene exposure to further increase the frequency of SCE and to elicit a significant level of chromatid-type aberrations in bone marrow cells of exposed animals (Table 1). This interaction between phenobarbital and benzene exhibited obvious sex differences with regard to both the type of the increased response and the magnitude of that response. Pretreatment with phenobarbital caused a further increase in SCE frequency in female but not in male mice exposed to benzene, and whereas chromosomal aberration yields were dramatically increased in both sexes, male mice had almost twice as many damaged cells as did females similarly treated with both agents.

Although the magnitude of the increase in SCE due to benzene exposure alone exhibited no sex dependency, inhalation of benzene significantly retarded proliferation of bone marrow cells in male but not in female DBA/2 mice (Fig. 1). This reduced replicative ability in male mice exposed to benzene is demonstrable by a significant shift in the proportions of first (I)-, second (II)-, and third (III)-generation metaphase cells observed in control animals toward a greater number of first-generation metaphase cells and fewer second- and third-generation metaphase cells. Furthermore, phenobarbital, which enhanced benzene's clastogenic activity in a sex-dependent fashion, also dramatically increased the inhibitory effect of benzene on cellular proliferation in a similar manner. Phenobarbital, by itself, caused a slight alteration in the rate of cellular proliferation in both male and female mice (Fig. 1). However, after benzene inhalation, phenobarbital-pretreated male mice exhibited almost a complete retardation of proliferative capacity. No cell had divided more than once in the 25 hr after benzene exposure, compared with a normal proliferative time for bone marrow cells under these conditions of approximately 10 hr per cell division (20). In female mice, the combination of benzene with phenobarbital pretreatment had no effect on the proliferative capacity of bone marrow cells.

Another set of experiments, designed to examine the induction of SCE at later times after exposure (i.e., 20–30 hr and 40–50 hr), again underscores the sex-dependent effect of benzene on bone marrow proliferative capacity (Fig. 2). With a 30-hr BrdUrd infusion, mice were infused either immediately after exposure to benzene or after approximately a 1-day delay. At both times, benzene-exposed male mice exhibited a significant inhibition of cellular proliferation when compared to control male or female mice or to exposed female mice (Fig. 2). However, the inhibition was much greater after the 1-day delay than that observed for the same period immediately after inhalation of benzene. In the first 30 hr after benzene inhalation, 78% of the metaphase cells had divided at least twice, whereas in an identical 30-hr period but after a 1-day delay only 1% of

Table 1. Effect of inhaled benzene on SCE frequency and chromosomal aberration yields in bone marrow cells of DBA/2 mice with and without phenobarbital pretreatment

Exposure group	Sex	SCE frequency	n*	Chromatid aberrations†			
				Achromatic lesions	Deletions	Iso deletions	Abnormal cells, %
Control	M	4.6 ± 0.2	6	0.068 ± 0.012	0.024 ± 0.012	0.004 ± 0.004	8.8 ± 1.2
	F	4.5 ± 0.3	6	0.092 ± 0.020	0.020 ± 0.012	0.004 ± 0.004	10.8 ± 2.2
Benzene	M	8.7 ± 0.4§	10	0.092 ± 0.016	0.046 ± 0.004	0.002 ± 0.002	12.8 ± 2.0
	F	8.2 ± 0.7§	11	0.100 ± 0.017	0.044 ± 0.008	0.000 ± 0.000	12.0 ± 1.3
Phenobarbital‡	M	4.1 ± 0.2	7	0.088 ± 0.018	0.028 ± 0.014	0.002 ± 0.002	10.8 ± 2.0
	F	4.2 ± 0.2	6	0.080 ± 0.014	0.020 ± 0.006	0.002 ± 0.002	9.6 ± 1.2
Phenobarbital‡ + benzene	M	8.3 ± 0.4	6	0.296 ± 0.038§	0.192 ± 0.020§	0.014 ± 0.004§	42.4 ± 3.6§
	F	12.7 ± 1.0§	7	0.216 ± 0.023§	0.096 ± 0.012§	0.012 ± 0.004§	25.6 ± 1.7§

Mean frequency per cell per animal ± SEM between animals is shown.
* Number of animals examined for SCE data.
† Data were derived from 5 animals in each group, 50 cells in each animal.
‡ Animals were injected with sodium phenobarbital (50 mg/kg) twice daily for 3 days prior to benzene exposure.
§ Statistically significant increase above appropriate control values at the 1% level.

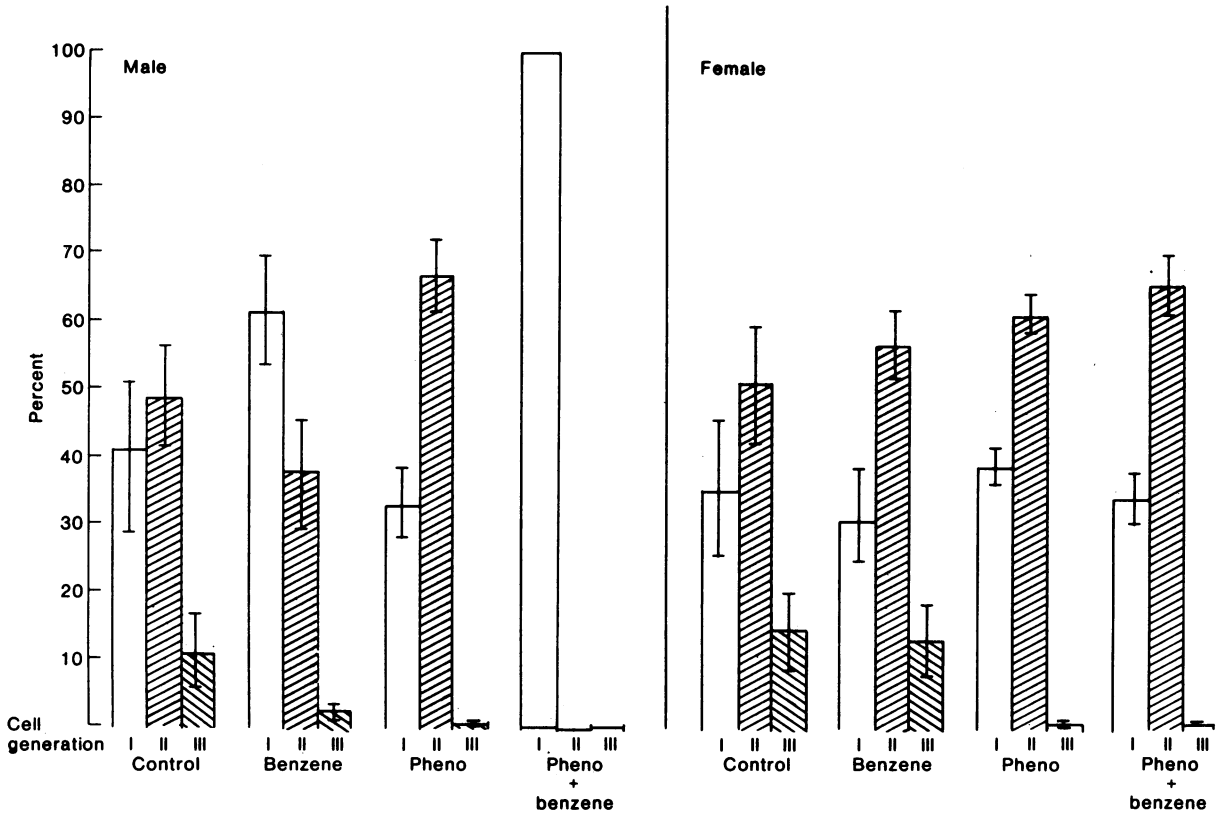

FIG. 1. Effect of inhaled benzene on cellular proliferation kinetics of bone marrow cells of DBA/2 mice, with and without phenobarbital (Pheno) pretreatment. The histograms present the replicative profile—that is, the relative proportions of metaphase cells that had divided for one (I), two (II), or three (III) generations during the BrdUrd infusion period. Range bars indicate SEM between animals. The number of animals in each treatment ranged from 5 to 12.

the metaphase cells had proliferated to this extent. These results support the observations of Lee *et al.* (21), which, based on the level of iron-59 incorporation into developing erythrocytes as a measure of benzene-induced bone marrow depression, also indicated increased depression of bone marrow activity during the second day after benzene exposure.

In an attempt to assess both the persistence and time-dependent formation of the benzene-induced lesions capable of eliciting an SCE response, we examined both the number and origin of SCE in metaphase cells that had replicated three times during a 30-hr infusion of BrdUrd in female mice killed 31 and 51 hr after benzene inhalation. Only female mice were used because their bone marrow proliferative activity remained normal after the exposure to benzene. Using these infusion durations and time periods we were able to compare the number of SCE induced during the first two BrdUrd replication cycles (≈ 1–21 and ≈ 21–41 hr) with those induced during the third BrdUrd replication cycles (≈ 21–31 and ≈ 41–51 hr) in bone marrow cells after benzene exposure. During each successive cell generation there exist two possible mechanisms for an increase in SCE above control levels: from new DNA lesions induced during that cell cycle or from DNA lesions induced during previous generations that had persisted as a consequence of a lack of repair. Determination of which mechanism is involved is based on a comparison of the number of SCE formed during the first two generations of BrdUrd infusion with those formed during the third generation (18, 22, 23). If all of the DNA lesions are induced prior to the first DNA synthetic period and no repair of this DNA damage takes place during subsequent replicative cycles, than the ratio of SCE formed during the first two cycles of BrdUrd incorporation to those SCE formed during the third cycle of BrdUrd incorporation will be 6:1, discounting background SCE. This ratio is based on the restriction of DNA damage to old DNA strands (i.e., strands free of BrdUrd substitution) and a subsequent dilution of these strands to daughter cells in a random fashion. If repair of these lesions does take place after their induction of SCE in the first BrdUrd incorporation cycle, preventing subsequent formation of SCE, then this ratio will become greater than 6:1; the magnitude of the increase depends directly on the efficiency of the DNA repair system(s) involved. Conversely, if new lesions are induced after the first BrdUrd incorporation cycle, then the ratio of first-generation plus second-generation SCE to third-generation SCE becomes less than 6:1. A combination of both new DNA lesions and persistence of old DNA lesions will also keep this ratio below 6:1. Our results (Table 2) suggest that both new lesions and persistence of old lesions occurred during the five generations of bone marrow cells examined in female mice after benzene exposure. SCE levels are still elevated above control values and, after background values are discounted, the ratios of first-generation plus second-generation SCE to third-generation SCE at both 31 and 51 hr after benzene exposure (2.0:1 and 1.4:1, respectively) are significantly lower than theoretical ratios derived for SCE occurring either as a result of persistent or of new lesions alone.

DISCUSSION

Although benzene is clearly both clastogenic and cytotoxic in this *in vivo* system, the relationship between the induced DNA damage and cellular inhibition remains unclear. First, both male and female DBA/2 mice exhibited increased numbers of SCE after exposure to benzene. Considering the suspected etiology of SCE induction, that of a replicative bypass mechanism permitting the progression of DNA synthesis in the pres-

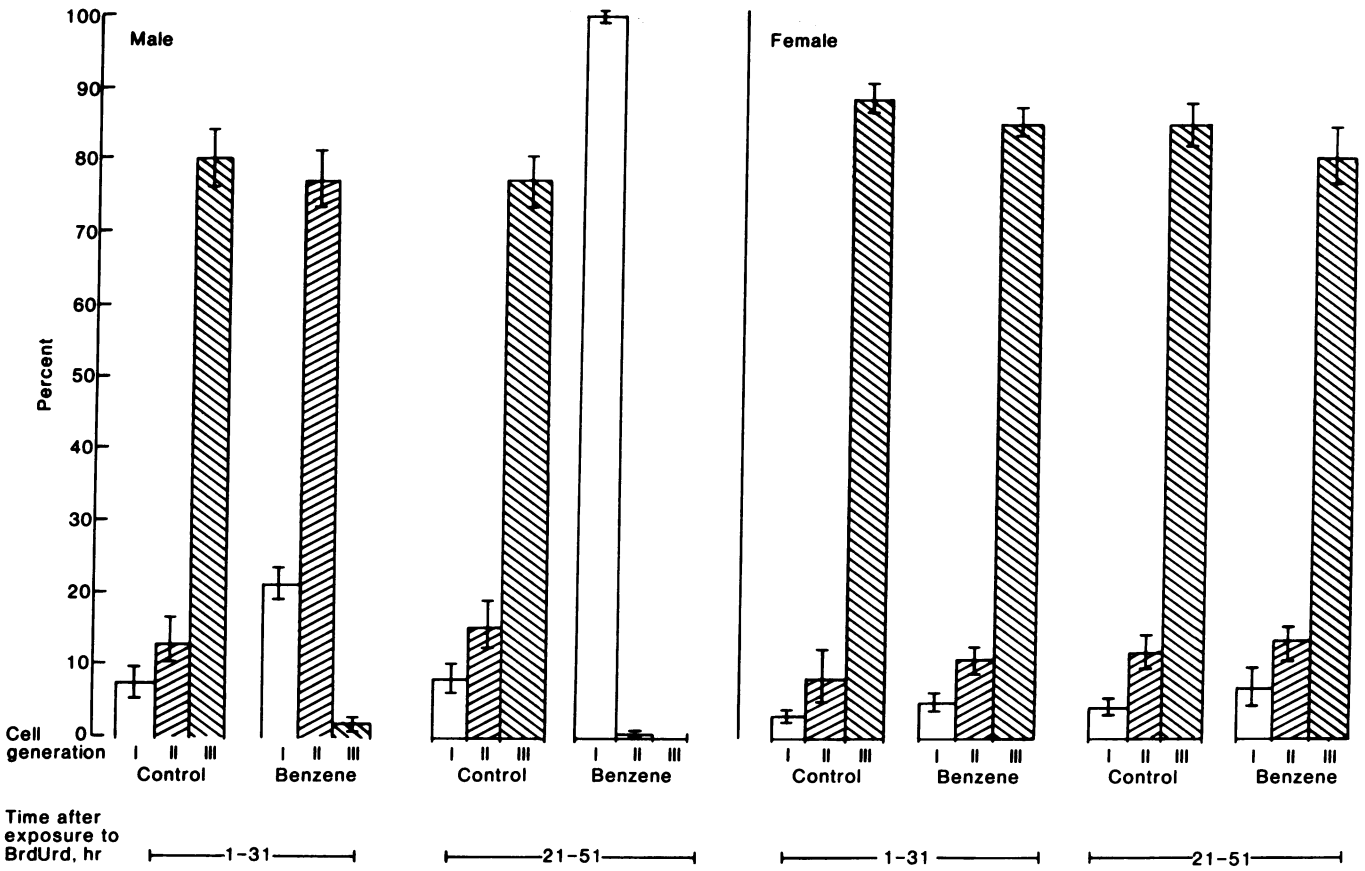

FIG. 2. Cellular proliferation kinetics in DBA/2 mice infused with BrdUrd either directly after benzene inhalation or after a 1-day delay. The histograms present the replicative profile—that is, the relative proportions of metaphase cells that had divided for one (I), two (II), or three (III) generations during the BrdUrd infusion period. Range bars indicate SEM between animals. The number of animals in each treatment was five or six.

ence of certain types and locations of lesions (24, 25), this increase presumably reflects the presence of an agent capable of damaging DNA to an equal extent in both sexes. However, only male mice exhibited inhibition of cellular proliferation and this inhibition occurred in the absence of a significant increase in chromosomal aberrations. Second, phenobarbital pretreatment clearly enhanced the induction of SCE, the induction of chromosomal aberrations, and the inhibition of cellular proliferation,

Table 2. Frequencies of symmetrical and asymmetrical SCE in third-generation metaphase cells of female DBA/2 mice examined 31 and 51 hr after benzene exposure

Time after benzene exposure, hr*	Exposure group	No. of animals	SCE† Asymmetrical	SCE† Symmetrical	Ratio A/S‡
31	Exposed	6	9.1 ± 0.3	3.4 ± 0.2	2.0:1
	Control	5	4.3 ± 0.3	1.0 ± 0.2	
51	Exposed	4	6.6 ± 0.2	2.8 ± 0.1	1.4:1
	Control	5	4.5 ± 0.3	1.3 ± 0.2	

* All animals were infused with BrdUrd for 30 hr prior to sacrifice.
† Mean value for SCE per cell per animal ± SEM between animals. Twenty-five third-generation metaphase cells were scored in each animal for the number of SCE induced in the first two generations of BrdUrd exposure (asymmetrical-appearing exchanges) and for the number of SCE induced in the third generation of BrdUrd exposure (symmetrical-appearing exchanges).
‡ Mean ratio of asymmetrical SCE to symmetrical SCE after subtraction of background.

suggesting that a metabolite(s), rather than benzene itself, was primarily responsible for these cytogenetic effects. However, because the various known metabolites of benzene were not actually measured in these experiments, it is not possible to definitely conclude whether phenobarbitol pretreatment altered the peak levels of certain metabolites by increasing the rate of benzene metabolism, altered the metabolic profile by inducing new pathways for benzene metabolism, or, in fact, interacted with benzene in a manner completely independent of phenobarbitol's known ability to enhance hepatic metabolism. The sex-related differences in both the type and magnitude of the observed responses suggest that phenobarbital is acting to alter benzene's metabolism and that different metabolites may have been responsible for the increases in these different cytogenetic endpoints. Although sex differences in the response of animals to benzene have also been reported for rats (26), there is no real evidence to suggest that female humans react differently from male humans when exposed to benzene (1–4).

We cannot conclude whether the proliferative inhibition observed in male mice exposed to benzene is due to an elongation of the cell cycle or to a reduced ability for cells to enter new generations. The restriction of chromosomal aberrations to chromatid type, as observed in this study and in an earlier study (27), is consistent with the type of damage induced by inhibitors of DNA synthesis (28). Because these agents also retard cell proliferation, our results may indicate a similar kind of interaction during the 2 days after benzene exposure in male mice. Yet female mice also exhibited an increase in this type of chromosomal aberration, but with no apparent decline in

bone marrow proliferative capacity. The increase in inhibition observed in male mice after a 1-day delay between benzene exposure and BrdUrd infusion may indicate either an increase in the level of a metabolite(s) of benzene capable of inhibiting cellular proliferation or a delayed response to an earlier damaging event(s). Previous studies based on the use of tritium-labeled benzene have concluded that metabolism and DNA binding in mice is essentially complete within 10-hr after benzene exposure (29). This observation suggests that the increased inhibition may result from a delayed response.

This study suggests that the relationship between exposure to benzene and its various hematological effects may be much more complex than was previously envisioned. It is also clear that the examination of only one cytogenetic end point in a single sex may lead to a rather naive interpretation of the data. Furthermore, the results presented here suggest that there is no *a priori* reason to assume some sort of common molecular mechanism among the induction of SCE, chromosomal aberrations, or cell cycle inhibition. The fact that male and female DBA/2 mice differed so greatly in both the magnitude and type of bone marrow response to benzene exposure or phenobarbital pretreatment (or both) suggests that this *in vivo* system may be extremely valuable in elucidating which metabolic pathways are responsible for which effects. Perhaps most important from the standpoint of the known leukemogenic properties of benzene is the observation that some of the DNA lesions induced by benzene exposure may persist through subsequent generations. Persistence of damage has been shown to correlate with carcinogenic induction in other systems (30, 31). The significance of this finding depends on a greater understanding of what types of lesions give rise to SCE and the relationship of these lesions to carcinogenesis.

We thank Dr. R. Snyder for helpful discussions and R. N. Ruffing for technical assistance. This paper was written under contract EY-76-C-02-0016 with the U.S. Department of Energy and the Environmental Protection Agency.

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