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Benzene Myeloclastogenicity: A Function of Its Metabolism

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Using the micronucleus test we have found no significant difference between germ-free and conventional (non-germ-free) male CD-1 mice gavaged twice with 440 or 880 mg benzene/kg. Hence. the higher myeloclastogenicity observed previously with the p.o. (4–6 times) than with the i.p. route of benzene administration was ruled out as being due to the involvement of gut flora in benzene biotransformation. Pretreatment of males with 3-methylcholanthrene or β-naphthoflavone. inducers of P 4 8 monooxygenase. but not phenobarbital. an inducer of P-450, significantly enhanced the myeloclastogenic effect of a single oral dose of benzene (440 mg/kg). Single oral doses of phenol, catechol. or hydroquinone (250. 150, and 200 mg/kg, respectively) failed to reproduce the potent myeloclastogenic effect of benzene. In fact, only hydroquinone was mildly clastogenic. The relation between benzene's myeloclastogenicity and metabolism is discussed.

Key words: chromosomal damage, micronucleus test in mice, hydroquinone, phenol, muconic acid, route of administration, 3-methylcholanthrene, phenobarbital, β-naphthoflavone, mixed function oxidase inducers

INTRODUCTION

The objective of our investigations on benzene has been to explore in vivo the relation between benzene's myeloclastogenicity and its metabolism, in order to elucidate the chemical pathogenesis of benzene-induced hemopathies. A "myeloclasrogen" (Greek *myelos*. medulla. marrow: Greek *klastos*, to break or *klasma*, a fragment; *genos*. birth) is defined as an agent which causes chromosomal breakage—including micronucleus formation—in the bone-marrow. Chromosomal aberrations have been

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reported in humans and animals following prolonged benzene exposure [Tough et al, 1970; Forni et al, 1971; Kissling and Speck, 1972a,b; Picciano, 1979]. We had previously established a dose-response relationship for benzene-induced myeloclastogenicity by both the micronucleus test (MNT) and metaphase chromosome analysis in CD-1 mice. Both tests were very sensitive to very low p.o. benzene dosage: at 8.8 mg/kg × 2 there was a doubling of damage effect over untreated controls ($p > 0.05$) and at 88 mg/kg × 2, a three- and fivefold increase in micronucleated polychromatic erythrocytes (MPCE) and aberrations per metaphase, respectively ($p < 0.05$). Moreover, pretreatment of the mice with 3-methylcholanthrene (3MC), a polycyclic aromatic hydrocarbon inducing a profile of hepatic microsomal activities different from that induced by phenobarbital (PB) [Meyer et al, 1980], greatly promoted benzene-induced myeloclastogenicity [Gad-El-Karim et al, 1984].

In CD-1 mice, the rates of micronucleated polychromatic erythrocytes were previously found to be 4–6 times higher with p.o. than i.p. benzene administration and the difference was statistically significant ($p < 0.01$) [Gad El Karim et al, 1984]. In this communication an experiment was designed to explore further this observed route difference of benzene administration using the MNT in germ-free mice. In addition, we compared the effects of the MFO inducers PB and 3MC vs. β-naphthoflavone (BNF), and the potent myeloclastogenicity of benzene vs. toxic doses of its known major metabolites, namely phenol (PH) [Babich and Davis, 1981], catechol (CA), and hydroquinone (HQ) [Nomiyama, 1965].

MATERIALS AND METHODS

Germ-free and conventional (ie, non-germ-free) male CD-1 mice, 7–10 weeks old and weighing 25–29 g, were purchased from Charles River Breeding Laboratories, Wilmington, MA. Germ-free animals were kept and treated in a sterile environment with artificial ventilation at 25° C, controlled humidity, and time-controlled lighting (12 hours of light per day). Regular microbial cultures of animal excreta, both aerobic and anaerobic, were performed by the company to confirm the germ-free status (being consistently negative) until the mice were treated by us and sacrificed. Conventional animals were allowed to acclimatize in cages under similar temperature, humidity, and lighting conditions. Mice were provided with food (Formulab chow 5008; Ralston Purina Co., St. Louis, MO) and water ad libitum. Three to five mice were randomly assigned to a treatment dose.

Chemicals

Benzene (thiophene free), phenol (PH), catechol (CA), and olive oil were obtained from Fisher Scientific Co., Fair Lawn, NJ. Cytoxan (cyclophosphamide, Mead Johnson Laboratories, Evansville, IN); sodium phenobarbital (PB) (St. Baker Chemical Co., Phillipsburg, NJ); hydroquinone (HQ), 3MC and BNF (Sigma Chemical Co., St. Louis, MO) were used in these experiments.

Pretreatments

The MFO inducers were administered i.p. (80 mg/kg per single dose) before the benzene: 3MC, 48 hours; BNF, 48 and 24 hours [Boobis et al, 1977], PB, 72, 48 and 24 hours. The 3MC and BNF were dissolved in olive oil and PB in distilled water (Gibco Laboratories, Grand Island, NY).

Treatment

Freshly prepared solutions of benzene in olive oil were formulated such that each animal received a volume of 0.01 ml/gm b.w., at 8 a.m. In experiment 1 the mice received two doses of benzene 24 hours apart and in experiment 2, one single dose. Animals were sacrificed 30 hours after the 1st dosing by cervical dislocation. One femur was immediately used for the MNT.

Micronucleus Test

The procedure was that of Schmid [1975] with some modifications [Connor et al, 1979; Gad-El-Karim et al, 1984]. (1) The bone marrow from one femur was flushed into a centrifuge tube containing a minimal amount of fetal calf serum (Gibco Laboratories). (2) Two slides were prepared from each cell button, and stained with Wright and Giemsa stains (Fisher Scientific Co.); the latter was diluted with pH 6.0 phosphate buffer. One thousand polychromatic erythrocytes (PCE) were scored and the number of the micronucleated ones (MPCE) recorded as per thousand of PCEs counted.

Statistical Analysis

The Mann-Whitney U-test was used to analyze the data; p-values were calculated for a two-tailed test. Values less than 0.05 were considered to be statistically significant. Data were presented with both the arithmetic mean ($\bar{X}$) and the geometric mean ($G.\bar{X}$)– the latter being more representative of a highly skewed group [Snedecor and Cochran, 1980].

RESULTS

Experiment 1 (Table I)

The differences in MPCE between germ-free benzene-treated or untreated control mice and those of the corresponding groups from conventional mice were statistically insignificant ($p > 0.05$). In either germ-free or conventional mice groups,

TABLE I. Micronucleus Formation Following Oral Benzene Administration in Germ-Free and "Conventional" Male CD-1 Mice*

Dosage	No. of mice	$\bar{X}$ ± S.D. of MPCE/1,000 PCE	G. $\bar{X}$ ± S.D. of MPCE/1,000 PCE	Range of MPCE/1,000 PCE
Control	3(G)	1.3 ± 1.5	1.4 ± 1.9	0-3[b]
(olive oil)	5(C)	3.2 ± 2.3	2.5 ± 2.4	1-6
Cytoxan	3(G)	36.7 ± 16.3	33.6 ± 1.7	18-48[a,b]
(50 mg/kg)	5(C)	36.4 ± 16.7	33.6 ± 1.6	21-62[a]
Benzene	3(G)	64.0 ± 24.2	61.0 ± 1.5	42-90[a,b]
(440 mg/kg)	5(C)	61.6 ± 26.2	56.6 ± 1.6	28-90[a]
Benzene	3(G)	49.3 ± 28.5	44.6 ± 1.7	30-82[a,b]
(880 mg/kg)	5(C)	60.0 ± 19.9	56.8 ± 1.5	30-82[a]

*Mice were treated twice, 24 hours apart, and sacrificed 30 hours after the first dosing.
$\bar{X}$ ± S.D., sample mean and standard deviation; G. $\bar{X}$, geometric mean; MPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; G, germ-free; C, Conventional (non-germ-free).
[a]Values significantly higher than control (olive oil) ($p < 0.01$) (Mann-Whitney U Test).
[b]Values statistically nonsignificant from those of conventional mice ($p > 0.05$).

benzene treatment (440 and 880 mg/kg) resulted in a statistically significant difference in MPCE from nontreated controls ($p < 0.01$).

Experiment 2 (Table II)

The 3MC and BNF—but not PB—pretreatment of benzene-treated mice, resulted in significantly higher MPCE than benzene treatment alone ($p < 0.01$). Moreover, 3MC scored higher MPCE than PB pretreatment, and the difference was statistically significant ($p < 0.05$).

The HQ treatment resulted in higher MPCE than untreated controls receiving the vehicle distilled water ($p < 0.01$); whereas, PH- and CA-treated mice showed virtually no difference from untreated controls.

Benzene Metabolites in Urine

We have developed a sensitive high pressure liquid chromatographic method (details of the methodology are published elsewhere, by Gad El-Karim et al [1985]) which yielded a well-resolved separation of urinary metabolites from benzene-treated male CD-1 mice. We have recovered *trans*, *trans*-muconic acid (MA) and HQ in the 48h urine collections in amounts representing each about 3% of the administered p.o. benzene dose (440 mg/kg b.w.), whereas PH accounted for 25% of the dose. Ninety-five percent of MA was recovered in the first 24 hours after benzene dosing.

DISCUSSION

The Micronucleus Test

The MNT which was positive with benzene in mice [Diaz et al, 1980; Hite et al, 1980; Meyne and Legator, 1980; Siou et al, 1981] has offered a unique endpoint for the study of benzene [Gad-El-Karim et al, 1984], since it is performed on cells

TABLE II. Micronucleus Test: Effect of Phenobarbital, 3-Methylcholanthrene, and β-Naphthoflavone Pretreatments on Benzene Myeloclastogenicity and Effects of Phenol, Catechol, and Hydroquinone Administration in Male CD-1 Mice[a]

Dose (p.o.) mg/kg	X ± S.D. of MPCE/1,000 PCE	G. X ± S.D. of MPCE/1,000 PCE	Range of MPCE/1,000 PCE
Control (olive oil)	0.6 ± 0.9	1.2 ± 1.4	0–2
Benzene (440)	35.6 ± 10.0	34.5 ± 1.3	24–49[b]
PB	1.4 ± 0.9	1.5 ± 1.5	0–2
PB + benzene (440)	55.8 ± 31.2	49.6 ± 1.7	24–107[b,c]
3MC	1.4 ± 0.9	1.5 ± 1.5	0–2
3MC + benzene (440)	110.2 ± 31.3	107.0 ± 1.3	77–161[b,c]
BNF	1.8 ± 1.3	1.8 ± 1.7	0–3
BNF + benzene (440)	85.4 ± 17.7	83.9 ± 1.2	63–104[b,c]
Control (distilled water)	0.6 ± 1.4	1.2 ± 0.9	0–2
Phenol (250)	1.0 ± 0.7	1.2 ± 1.4	0–1
Catechol (150)[d]	1.2 ± 1.1	1.5 ± 1.5	0–2
Hydroquinone (200)	10.0 ± 2.2	9.8 ± 1.3	7–13[b]

[a]Each group comprised five animals.
[b]Values significantly higher than untreated controls ($p < 0.01$).
[c]Values significantly higher than benzene treatment alone ($p < 0.01$).
[c]Values significantly lower than the 3MC + benzene (440)-treated group ($p < 0.05$).
[d]One animal died from the convulsive seizures which immediately followed dosing.

originating in the bone marrow, the site of benzene toxicity [Laskin and Goldstein, 1977; Snyder et al, 1977]. The MNT reveals early insult to erythropoiesis reflected in the maturing erythrocytes [Matter et al, 1974; Schmid, 1975; Von Ledebur and Schmid, 1973]. The low and consistent spontaneous rate of micronuclei in the marrow (about 3 MPCE per 1,000 PCE) as well as the easiness of identifying the PCE render both the numerator and denominator of this rate reproducible and obtainable, respectively. In comparison, peripheral blood counts exhibit wide variations in normally observed hematologic parameters, as Leong [1977] pointed out ". . . nearly all previous reports on leukopenia in animals exposed to less than 1,000 ppm benzene are unlikely to stand statistical evaluation." Micronuclei are synonymous with Howell-Jolly bodies that are found in man [Larrimer et al, 1975] mainly after splenectomy, in pernicious anemia, leukemia, and erythroleukemia or DiGuglielmo syndrome—a rare entity which has been linked with benzene toxicity [Forni and Moreo, 1969; Kohly et al, 1967; Rozman et al, 1968].

Effect of the Route of Benzene Administration on Micronucleus Formation

The rates of MPCE in mice have been found to be higher following the p.o. than the i.p. route of benzene administration [Meyne and Legator, 1980; Gad-El-Karim et al, 1984]. Moreover, 3MC pretreatment greatly enhanced the myeloclastogenicity of p.o. rather than i.p. administered benzene [Gad El-Karim et al, 1984].

In this communication, the p.o. route was studied using germ-free vs. "conventional" (non-germ-free) male CD-1 mice in order to determine whether the gut flora could have had a role in the biotransformation of benzene [Gabridge et al, 1969; Batzinger et al, 1978; Tunek et al, 1978] and accounted for the more potent myeloclastogenicity observed with the p.o. route. This possibility is ruled out since no significant difference was observed (Table I). We believe that benzene absorption into the portal circulation occurs more rapidly from the highly vascular visceral peritoneum following i.p. injection [Lukas et al, 1971] than from the lumen and epithelial wall of the intestines after p.o. administration, resulting in increased loss of unmetabolized benzene via the lungs. Therefore, not as many dividing marrow cells would be exposed to the clastogenic effect of benzene metabolite(s) due to the limited availability of the compound for biotransformation after i.p. injection. Following p.o. and i.p. benzene administration in the rat, Gut [1976] reported low benzene concentrations that rapidly declined in both mixed venous blood and liver. The author ascribed these observations to relatively high volumes of distribution of benzene (5.4–5.6) in addition to its rapid elimination (unchanged) by the lungs, and *not* to rapid metabolism. On the other hand, the role of intestinal MFO in metabolizing p.o. administered benzene remains undetermined.

Sex Difference With Benzene

Male mice were reported to be more sensitive to benzene induction of chromosomal damage than females [Meyne and Legator, 1980; Siou et al, 1981; Gad-El-Karim et al, 1984] and pregnant females were even more resistant to benzene [Gad-El-Karim et al, 1984]. Upon treatment with testosterone, castrated males, as sensitive as females, regained their original sensitivity [Siou et al, 1981]; thus, hormonal factors were ascribed to female resistance to benzene.

In a study on human workers exposed to benzene, Hunter [1939] cast "considerable doubt on female hypersusceptibility." Only two of ten fatal cases in his series

were females and, though the only fatal case in one factory was a young girl, of the workers examined from this same factory, the men formed 60.5% and furnished 64% of the cases, and three out of seven females and only one out of 26 males had normal blood pictures. Correlation of the marrow status with sex and duration of exposure together, revealed that hyperplasia, seen only with a minimum of 2 years exposure, was distinctly more common in males than hypoplasia which occurred with less than 1 year exposure. Hypoplasia was more common in females irrespective of the duration of exposure. Nevertheless, severe anemia could be present with either reaction [Mallory et al, 1939]. Interestingly, in a prospective study on 53 patients with "aregenerative" anemia—irrespective of the etiology—acute leukemia was significantly more common in patients with cytopenia and a normal or hypercellular marrow than in those with a hypocellular marrow [Hast et al, 1976].

Effect of Modifiers of MFO Activity on Benzene Myeloclastogenicity

Pretreatment with PB protected rats from benzene-induced leukopenia [Ikeda and Ohtsuji, 1971; Drew et al, 1973; Gill et al, 1979] but 3MC pretreatment did not [Gill et al, 1979]. Hirokawa and Nomiyama [1962] observed that old rats, which metabolized benzene more slowly than the younger ones, were less susceptible to benzene toxicity. Nomiyama [1962, 1964] reported that 3-amino-1,2,4-triazole inhibited benzene metabolism in vitro, by rat liver homogenates, as well as in vivo, where it alleviated benzene-leukopenia in rats. 3-amino-1,2,4-Triazole also abolished benzene-induced inhibition of ^{59}Fe incorporation into developing erythrocytes [Bolesak and Nerland, 1983]. Hence, a paradox existed: both inhibitors and inducers of benzene metabolism protected against toxicity. Thus, it has been postulated that inducers stimulate hepatic detoxification, whereas inhibitors prevent the formation of reactive metabolites in the bone marrow [Snyder et al, 1977].

In our studies, PB pretreatment did not protect CD-1 mice against benzene-induced myeloclastogenicity [Gad-El-Karim et al, 1984]; nevertheless, individual animals of the same group exhibited a wider range of MPCE and a higher mean, though statistically insignificant, than mice receiving benzene alone (Table II). This is in agreement with previous observations where PB did not protect against benzene-induced depression of ^{59}Fe uptake into mouse erythrocytes [Snyder, 1973], despite its protection against rat leukopenia. Pretreatment with 3MC as well as BNF (Table II), inducers of P-448 monooxygenase, significantly enhanced benzene's myeloclastogenicity ($p < 0.01$). Neither Aroclor 1254 nor SKF-525A pretreatments had a significant effect [Gad-El-Karim et al, 1984].

Effect of Benzene Metabolites on Micronucleus Formation

In the present study, very toxic single doses of PH, CA, or HQ were given p.o. to male mice. Severe convulsive seizures immediately followed dosing in each animal. Neither PH nor CA induced micronuclei in the bone marrow. The HQ exhibited a mild clastogenic activity as compared to benzene (Table II). Using 150 mg/kg × 2 p.o. doses of PH, CA, or HQ, with and without 3MC pretreatment, further proved that only HQ was mildly clastogenic, by both the MNT and metaphase chromosome analysis, and that 3MC pretreatment, in particular, had no effect [Gad-El-Karim, unpublished]. It is possible, nevertheless, that the elimination kinetics or the distribution of the metabolites are substantially different when they are formed from benzene in vivo, as opposed to being administered p.o. Tunek et al [1982] used

subcutaneous doses ranging between 20 and 160 mg HQ/kg b.w./day × 6 and reported micronuclei at all doses above 6 × 20 mg/kg b.w.

Benzene Metabolites in Urine-Including *trans, trans*-Muconic Acid

The existence of MA was previously confirmed by Parke and Williams [1953a], who administered ^{14}C-benzene orally to rabbits and recovered 1.0–1.8% of the benzene dose (0.4–0.34 g/kg, respectively) as MA in urine. The finding of MA in CD-1 mouse urine suggests a hitherto unconfirmed toxic metabolic pathway. That pathway involves the opening of the benzene ring [Goldstein et al, 1981]. Interestingly, *none* of the p.o. dosed groups of mice which received individual metabolites of benzene, namely PH, CA, or HQ, exhibited any trace of MA in their urine [Gad-El-Karim et al, 1985]. Hence, the production of MA was unique to benzene. None of the three administered metabolites was subjected to ring opening. This confirms the previous findings by Parke and Williams [1953b], who reported that neither PH nor CA were precursors of MA in the rabbit.

CONCLUSIONS

Based on our findings in mice [Gad-El-Karim et al, 1984, 1985], we have drawn the following conclusions and postulations.

1. The myeloclastogenic effect of benzene appears to be the earliest benzene-induced cellular damage in which chromosomal DNA is a primary critical target, and which is readily identifiable (scorable), and antedates changes in peripheral blood counts (leucocytic, platelets counts, etc).

2. Benzene's myeloclastogenicity is a function of its metabolism. Modification of benzene metabolism by an appropriate MFO inducer as 3MC or BNF or the co-administration of toluene is reflected in the extent of chromosomal damage, being increased or decreased, respectively.

3. Female mice were consistently more resistant to benzene than males; interestingly, pregnant females were the most resistant.

4. Benzene myeloclastogenicity is greater with the p.o. than with the i.p. route of administration.

5. Bacterial flora did not account for the superiority of the oral route.

6. The following points may be significant in interpreting the mechanism of benzene myeloclastogenicity: a) Enhancement of chromosomal damage by P-448 rather than P-450 enzyme inducers. b) HQ, CA, or PH do not possess any of the potent myeloclastogenicity of the parent compound, benzene. HQ, in fact, shows a mild clastogenic response at a toxic dose (200 mg/kg b.w.). c) The unique presence of *trans,trans*-muconic acid in the urine of benzene-treated mice and *not* of those animals treated with HQ, CA, or PH.

Based on the above findings, we postulate that the likely events involved in the biotransformation of benzene entail the reported ability of benzene to induce its own metabolism [Snyder et al, 1967], described as being similar to that of the polycyclic aromatic hydrocarbons [Gonasun et al, 1973; Saito et al, 1973]. This is confirmed by the P-448 inducers 3MC and BNF, which induce a critical pathway [Haughen et al, 1976; Tunek and Oesch, 1979; Dresner et al, 1981; Nebert, 1981; Nebert et al, 1981] of benzene metabolism in mice that leads to enhanced myeloclastogenicity. Induction of bone-marrow MFO [Andrews et al, 1979; Irons et al, 1980], especially the nuclear

aryl hydrocarbon hydroxylase, could play a significant role in covalent binding of metabolites to nuclear DNA [Jernström et al, 1976; Gill and Ahmed, 1981; Lutz and Schlatter, 1977], especially in a rapidly proliferative organ such as bone marrow. The induction of epoxide hydrase may well be an additional important factor. Benzene oxide [Jerina et al, 1968; Tunek et al, 1978], which proved to be a potent inducer of DNA damage [Kinoshita et al, 1981] and/or a putative aldehyde precursor of muconic acid capable of alkylating DNA, muconaldehyde [Goldstein et al, 1981], could be the likely candidates playing the major role in the chemical pathogenesis of benzene-induced chromosomal damage, aplastic anemia, preleukemia, and leukemia [Högstedt and Mitelman, 1981]. Hydroquinone's role, however, can only be ancillary and possibly more significant in chronic exposure, inasmuch as only a small amount is produced from benzene.

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