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In Vitro Effects of Benzene Metabolites on Mouse Bone Marrow Stromal Cells

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In Vitro Effects of Benzene Metabolites on Mouse Bone Marrow Stromal Cells. GAIDO, K., AND WIERDA, D. (1984). *Toxicol. Appl. Pharmacol.* 76, 45–55. Benzene exposure can result in bone marrow myelotoxicity. We examined the effects of benzene metabolites on bone marrow stromal cells of the hemopoietic microenvironment. Male B6C3F$_1$ mouse bone marrow adherent stromal cells were plated at 4×10^6 cells per 2 ml of DMEM medium in 35-mm tissue culture dishes. The growing stromal cell cultures were exposed to log 2 doses of five benzene metabolites: hydroquinone, benzoquinone, phenol, catechol, or benzenetriol for 7 days. The dose which caused a 50% decrease in colony formation (TD50) was 2.5×10^{-6} M for hydroquinone, 17.8×10^{-6} M for benzoquinone, 60×10^{-6} M for benzenetriol, 125×10^{-6} M for catechol, and 190×10^{-6} M for phenol. We next examined the effect of benzene metabolites on the ability of stromal cells to influence granulocyte/monocyte colony growth (G/M-CFU-C) in a coculture system. Adherent stromal cells were plated and incubated for 14 days and then exposed to a benzene metabolite. After 3 days the medium and metabolite were removed and an agar:RPMI layer containing 10^6 fresh bone marrow cells was placed over the stromal layer. After incubation for 7 days the cultures were scored for G/M colony formation. Hydroquinone and benzoquinone were most toxic, while catechol and benzenetriol inhibited colony growth only at high doses. These results indicate that injured bone marrow stromal cells may be a significant factor in benzene-induced hemotoxicity.

Chronic exposure to benzene can lead to a wide range of hematological disorders including leukopenia, lymphocytopenia, acute myelogenous leukemia, and aplastic anemia (Laskin and Goldstein, 1977; Cohen *et al.*, 1978; Snyder *et al.*, 1981). Benzene metabolism is important for the expression of hematotoxicity. Either the inhibition of the benzene-metabolizing enzymes (Andrews *et al.*, 1977; Rickert *et al.*, 1979, 1981) or the induction of benzene metabolism (Greenlee and Irons, 1981; Wierda *et al.*, 1981) will lead to a decrease in toxicity due to benzene, which suggests that an intermediate metabolite is responsible for disorders associated with benzene exposure. Studies by Tunek *et al.* (1981) have demonstrated that an increase in benzene toxicity to an organ is associated with an increase in benzene metabolites in that organ. In addition Morimoto and Wolff (1980) have determined that several benzene metabolites are more potent than benzene in inducing sister chromatid exchange.

Metabolites of benzene include phenol, hydroquinone, and catechol (Rickert *et al.*, 1981; Longacre *et al.*, 1982; Greenlee and Irons, 1981; Tunek and Oesch, 1982). Hydroquinone and catechol may undergo further oxidation to produce benzosemiquinone and benzoquinone (Tunek *et al.*, 1980; Irons *et al.*, 1982; Tunek and Oesch, 1982; Sawahata and Neal, 1983). It is not known which benzene metabolite causes the ultimate toxicity but hydroquinone and catechol concen-

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trate in the bone marrow (Rickert *et al.*, 1979, 1981; Greenlee *et al.*, 1981) where these metabolites may be further metabolized and affect blood forming cells (Irons *et al.*, 1980, 1982; Andrews *et al.*, 1979; Johansson and Ingelman-Sundberg, 1983; Bolcsak and Nerland, 1983).

Current evidence suggests that benzene is preferentially cytotoxic to the maturing blood precursor cells within the bone marrow. Benzene cytotoxicity has been demonstrated toward progenitor cells of the lymphocyte, erythrocyte, granulocyte, and monocyte cell classes (Lee *et al.*, 1974; Tunek *et al.*, 1981; Wierda and Irons, 1982; Pfeiffer and Irons, 1982). Work by Irons *et al.* (1979) has shown a preferential cytotoxicity of benzene toward progenitor blood cells of intermediate differentiation while sparing the mature, nondividing cell types.

Hemopoietic stem cells *in vivo* undergo the process of differentiation and maturation in direct association with specialized stromal cells which make up the hemopoietic microenvironment (Metcalf and Moore, 1971; Dexter, 1983). The major site of blood cell formation is within the bone marrow. Bone marrow stromal cells form a supporting matrix for the developing cells and may directly influence and regulate hemopoietic cell development (Lichtman, 1981; Zipori, 1981; Bentley, 1982). In culture, bone marrow stromal cells form colonies which consist of fibroblast and macrophage cells which adhere to the culture dish (Friedenstein *et al.*, 1976; Werts *et al.*, 1980; Zipori and Bol, 1979; Wiktor-Jedrzejczak *et al.*, 1981). A coculture system, in which adherent stromal cells are in contact with an over layer of medium containing hemopoietic precursor cells, is an *in vitro* model of the hemopoietic microenvironment. In this culture system the adherent stromal cells induce and regulate hemopoiesis (Reimann and Burger, 1979; Dexter *et al.*, 1977, 1982; Bentley, 1981; Zipori, 1981).

Knowledge of benzene effects on bone marrow stromal cells is limited. It is not clear what effect benzene has on the bone marrow stromal cells. Toxicity of benzene toward the stromal cells could disrupt the hemopoietic microenvironment and interrupt normal hemopoiesis resulting in the hemotoxicity associated with benzene exposure. The purpose of our study was twofold. First, with liquid bone marrow cultures, we determined the dose–response effect of several benzene metabolites on adherent stromal cell colony formation (a measure of cell survivability and proliferating capability in the presence of metabolite). Second, we used a coculture system of metabolite exposed stromal cells and fresh bone marrow cells in agar to determine whether benzene metabolites could alter the ability of stromal cells to support hemopoiesis of precursor cells; specifically granulocyte/monocyte colony-forming cells. Results from these studies were also used to gain a perspective on the most likely metabolite or metabolites responsible for hematotoxicity associated with benzene exposure.

METHODS

Mice. Male B6C3F$_1$ mice, 6 to 10 weeks old when used in our study, were obtained from Charles River Breeding Laboratories, Wilmington, Mass. The mice were housed four to a cage with hardwood bedding and had free access to feed (Wayne Centrified Lab Blox, Allied Mills, Inc., Chicago, Ill.) and acidified water (pH 2.5 to 3.0). A 12 hr light cycle was maintained (0700 to 1900 hr).

Metabolites. The benzene metabolites, hydroquinone (Sigma Chemical Co., St. Louis, Mo.), benzoquinone (Aldrich Chemical Co., Milwaukee, Wisc.), catechol (Sigma), phenol (Fisher Scientific Co., Pittsburgh, Pa.), and 1,2,4-benzenetriol (Aldrich) were diluted to the indicated concentrations in pyrogen-free saline (Travenol, Chicago, Ill.). Solutions of these compounds were prepared fresh immediately before addition to cell cultures.

Bone marrow cell suspensions. Mice were killed by cervical dislocation and their femurs and tibias removed. The bone marrow cells were flushed from the marrow cavities and pooled in 5 ml of collecting medium in a siliconized 35-mm glass Petri dish. The collecting medium was Hanks' basic buffer solution (Whittaker Bioproducts, Walkersville, Md.) with 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma). The bone marrow was aspirated several times with a Pasteur pipet to suspend the cells. Cell number was determined by counting nucleated cells with a hemacytometer.

Bone marrow adherent stromal cell cultures. The procedure employed was a modification of the method of Zipori and Bol (1979). The bone marrow cell suspension was adjusted to a concentration of 2×10^6 cells/ml by dilution with DMEM supplemented with 7.5% fetal calf serum and 7.5% horse serum (Whittaker Bioproducts) and with 2 μM glutamine, 5×10^5 M 2-mercaptoethanol, 100 g/ml L-asparginine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma). The cell suspension (2 ml) was placed in 35-mm plastic Petri dishes (Falcon 2001) in duplicate for control and for each dose. When indicated the appropriate concentration of benzene metabolite was also added (0.2 ml/2 ml culture). The cultures were incubated at 37°C in an atmosphere of 5% CO_2 and air. On Day 3, the medium and suspended cells were removed, and the cultures were reconstituted with 2 ml of fresh DMEM plus the appropriate benzene metabolite. Removal of nonadherent cells on Day 3 results in greater numbers of colonies since these nonadherent cells presumably exert a feed-back inhibition on stromal cell growth if they remain in the cultures. The stromal cell cultures were terminated on the seventh day by removal of the medium and fixed with Wright–Giemsa stain. The colonies were scored on an inverted microscope.

Bone marrow coculture system. The procedure used is a modification of the method reported by Zipori (1981). The bone marrow cell suspension was adjusted to a concentration of 2×10^6 cells/ml in RPMI-1640 (Whittaker Bioproducts) supplemented with 7.5% fetal calf serum and 7.5% horse serum (Whittaker Bioproducts) plus 2 μM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml Na pyruvate, and 5×10^{-5} M 2-mercaptoethanol (Sigma). The cell supsension was placed in 35-mm plastic Petri dishes (Falcon #3001) (2 ml/dish) in triplicate for control and for each dose. The cell cultures were incubated at 37°C in 5% CO_2 and air for 14 days. The cultures were refed by a complete replacement of RPMI medium on Day 3 and a 50% replacement of medium on Day 7 and on Day 14. After the medium replacement on Day 14, the indicated benzene metabolite was added (0.2 ml/2 ml of medium). On Day 17, the medium was removed and 0.5 ml of a fresh bone marrow suspension (2×10^5 cells/ml of medium) plus a 0.5 ml mixture of 2 × RPMI: 6% bacto agar was added over the adherent layer. The 2 × RPMI was supplemented with 20% FBS, 10% HS plus 2 μg/ml DEAE-dextran, 11.2 mM glucose, 200 IU/ml penicillin, 200 μg/ml streptomycin, 200 μg/ml Na pyruvate, 2 mM glutamine, and 10^{-4} M 2-mercaptoethanol. The medium agar mixture was allowed to gel and then incubated for an additional 7 days. On Day 24 the number of granulocyte/monocyte colonies per culture was counted with an inverted microscope.

Statistics and computations were done with an Apple computer with programs by Tallarida and Murray (1981). Probit analysis for all dose response curves were done to determine TD50s as a means of comparison of relative toxicity. Statistical significance for all assays were determined at the 5% level of significance by Dunnett's *t* test.

RESULTS

Effect of Benzene Metabolites on Adherent Stromal Cell Colony Formation

To determine the dose–response effect of benzene metabolites on bone marrow stromal cells we added increasing concentrations of metabolites to adherent stromal cell cultures. The ability of stromal cells to survive and proliferate was determined by counting the number of adherent stromal cell colonies formed in the presence of log 2 doses of benzene metabolite (1.5, 3.1, 6.2, 12.5, 25, 50, and 100×10^{-6} M). Colony number at each dose was determined and compared with control values from stromal cell cultures which did not receive any metabolite. The values from duplicate cultures were expressed as a percent of control (Figs. 1 to 5), and each experiment was performed twice on separate days.

Phenol at most doses had very little effect on the number of stromal cell colonies formed (Fig. 1). At 100×10^{-6} M, the highest dose

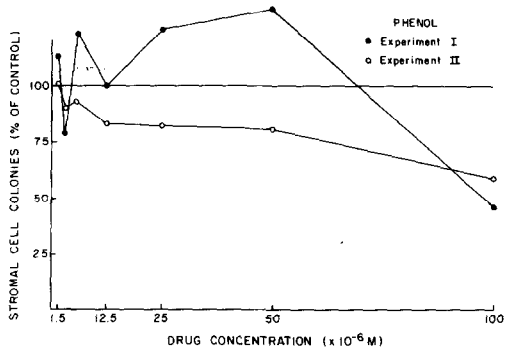

FIG. 1. Effect of phenol on adherent stromal cell colony formation. Mouse bone marrow cells were incubated for 7 days in the presence of phenol. Values represent mean stromal cell colony formation for duplicate cultures and are expressed as percentages of control. Control values averaged 26 ± 4 for experiment I and 80 ± 7 for experiment II. Standard errors for the metabolite exposed cultures did not exceed 13% of mean values.

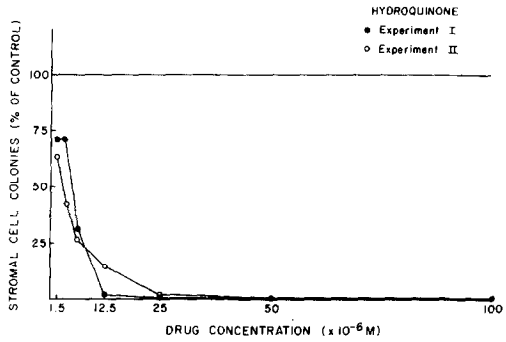

FIG. 2. Effect of hydroquinone on adherent stromal cell colony formation. Mouse bone marrow cells were incubated for 7 days in the presence of hydroquinone. Values represent mean stromal cell colony formation and are expressed as percentages of control. Control values averaged 68 ± 11 for experiment I and 109 ± 2 for experiment II. Standard errors for the metabolite exposed cultures did not exceed 26% of mean values.

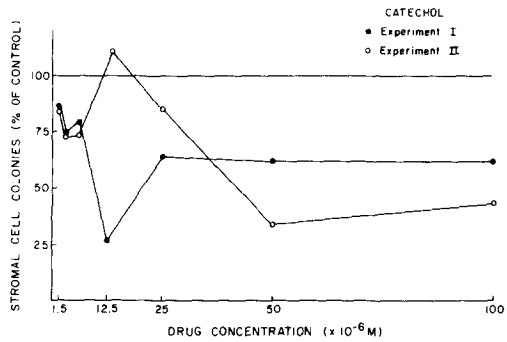

FIG. 4. Effect of catechol on adherent stromal cell colony formation. Mouse bone marrow cells were incubated for 7 days in the presence of catechol. Values represent mean stromal cell colony formation and are expressed as percentages of control. Control values averaged 33 ± 2 for experiment I and 66 ± 7 for experiment II. Standard errors for the metabolite exposed cultures did not exceed 18% of mean values.

used, phenol caused a 42 to 54% decrease in the number of colonies formed. Addition of hydroquinone to the adherent stromal cell culture system caused a significant dose-related decrease in colony formation (Fig. 2). At the lowest dose of 1.5×10^{-6} M, hydroquinone caused a 29 to 37% decrease in colony number. Colony formation was completely inhibited at doses of 50 and 100 $\times 10^{-6}$ M hydroquinone. Benzoquinone, the oxidation product of hydroquinone, also had a dose-related effect on the adherent stromal cell colonies (Fig. 3). Higher doses of benzoquinone were required than for hydroquinone to cause an effect, but colony formation was completely inhibited at a dose of 100 $\times 10^{-6}$ M. Catechol had little effect on the stromal cell colony formation at lower doses

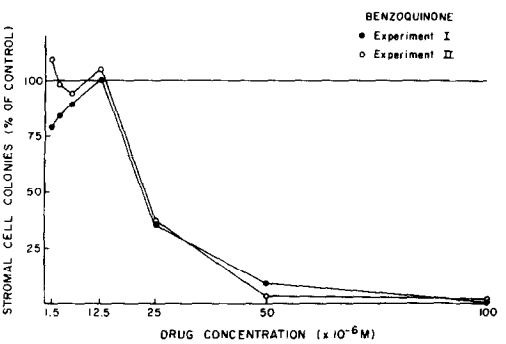

FIG. 3. Effect of benzoquinone on adherent stromal cell colony formation. Mouse bone marrow cells were incubated for 7 days in the presence of benzoquinone. Values represent mean stromal cell colony formation and are expressed as percentages of control. Control values averaged 70 ± 8 for experiment I and 106 ± 10 for experiment II. Standard errors for the metabolite exposed cultures did not exceed 39% of mean values.

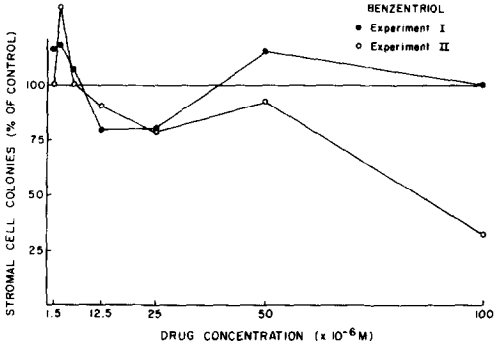

FIG. 5. Effect of benzenetriol on adherent stromal cell colony formation. Mouse bone marrow cells were incubated for 7 days in the presence of benzenetriol. Values represent mean stromal cell colony formation and are expressed as percentages of control. Control values averaged 79 ± 6 for experiment I and 31 ± 5 for experiment II. Standard errors for the metabolite exposed cultures did not exceed 30% of mean values.

(Fig. 4). At a dose of 50×10^{-6} M catechol there was a 40 to 60% decrease in colony formation. Increasing the dose to 100×10^{-6} M did not cause any further decrease in stromal cell colony formation. Benzenetriol, an oxidation product of catechol, had a variable effect on the stromal cell culture system (Fig. 5). At a dose of 3.1×10^{-6} M benzenetriol induced a 15 to 35% increase in colony formation. Further increases in the dose of benzenetriol resulted in colony numbers equal to control values. At the highest dose of 100×10^{-6} M values from the two benzenetriol experiments differed from a 0 to 68% decrease in colony formation.

For a comparison of the relative toxicity of these metabolites the data from Figs. 1 to 5 were applied to probit analysis (Table 1), and the toxic dose at which there was a 50% decrease in colony formation based on control values (TD50) was determined. Based on a comparison of probit plots for each of the metabolites, hydroquinone was most toxic to the stromal cells with a TD50 of 2.2 to 2.95 $\times 10^{-6}$ M. Benzoquinone was slightly less toxic followed by benzenetriol, catechol, and phenol. In some instances, especially with the less toxic metabolites, benzenetriol and phenol, the data did not fit a dose–response relationship and thus an accurate probit analysis could not always be done to determine from TD50s for these experiments. For catechol and phenol the TD50s were beyond the dose range tested and are therefore only projected TD50s.

TABLE 1

ESTIMATED TD50 OF BENZENE METABOLITES ON PERCENTAGE STROMAL CELL COLONY FORMATION[a]

Benzene metabolite		Probit analysis	
		TD50 ($\times 10^{-6}$ M)[b]	Slope
Hydroquinone	Experiment I	2.20 ± 1.6	1.98
	Experiment II	2.95 ± 4.8	2.05
Benzoquinone	Experiment I	18.90 ± 23.7	2.13
	Experiment II	16.46 ± 15.3	2.09
Benzenetriol	Experiment I	—	—
	Experiment II	59 ± 117.5[c]	3.20
Catechol	Experiment I	129.20 ± 807.5[c]	4.88
	Experiment II	119.70 ± 772[c]	4.97
Phenol	Experiment I	189 ± 255.5[c]	7.41
	Experiment II	—	—

[a] Cells were cultured in duplicate in the presence of a benzene metabolite for 1 week. Experiments were performed on separate days.

[b] TD50 = dose at which there was a 50% reduction in colony formation compared to control values.

[c] Projected TD50.

Effect of Benzene Metabolites on the Ability of Stromal Cells to Influence Hemopoiesis

A coculture system was used to examine the ability of bone marrow stromal cells to influence normal hemopoiesis. After exposure to benzene metabolite, stromal cell function was determined by the number of granulocyte/monocyte colonies formed in the agar layer in triplicate cultures as compared with control values in which the adherent stromal cells were not exposed to metabolite.

Stromal cell exposure to hydroquinone had a significant dose related effect on G/M colony formation (Table 2). A dose of 1.5×10^{-6} M hydroquinone caused a 21 to 33% decrease in the number of G/M colonies in the agar layer. At a dose of 6.2×10^{-6} M G/M colony formation decreased 65 to 66% compared to control values. Benzoquinone also showed a significant dose related effect on G/M colony formation (Table 3). A dose of 3.1×10^{-6} M caused a 32 to 50% decrease in colony number. At the highest dose of 25×10^{-6} M, benzoquinone decreased the number of G/M colonies 63 to 100% of control. High doses of catechol (25 to 200×10^{-6} M) were needed to affect stromal cell influence on G/M colony formation (Table 4). As the dose of catechol was increased from 25×10^{-6} to 200×10^{-6} M colony number decreased significantly to 2 to 11% of control values. Benzenetriol had very little effect on granulocyte/monocyte colony formation at low doses (Table 5), only at high doses of 100 to 500×10^{-6} M was there a significant inhibi-

TABLE 2

EFFECT OF HYDROQUINONE ON THE ABILITY OF ADHERENT STROMAL CELLS TO INFLUENCE G/M-CFU-C GROWTH[a]

Dose ($\times 10^{-6}$ M)	Experiment I[b]	Experiment II[b]
Control	20.3 ± 2	37 ± 8.5
1.5	16 ± 2 (79%)[c]	25 ± 2 (67.5%)
3.1	16.3 ± 2 (80%)	16 ± 3[d] (43%)
6.2	7 ± 1[d] (34%)	13 ± 3[d] (35%)
12.5	11.6 ± 1[d] (57%)	6 ± 2[d] (16%)

[a] Adherent stromal cell colonies were grown for 14 days and then exposed to the indicated hydroquinone dose for 3 days, after which time the medium was removed and an agar layer was plated over the adherent stromal cells and cultured for 7 days.

[b] Values represent the mean of triplicate cultures ± SE. Experiments were performed on separate days.

[c] Numbers in parentheses represent percentage of control.

[d] Significantly different from control, $p < 0.05$.

tion of colony formation. The decreases in granulocyte/monocyte colony formation corresponded closely with a decrease in the adherent stromal cell colony number in each coculture experiment (data not shown).

Without an adherent stromal cell layer, no colonies were produced. Addition of L929 colony stimulating factor (CSF) to cultures

TABLE 3

EFFECT OF BENZOQUINONE ON THE ABILITY OF ADHERENT STROMAL CELLS TO INFLUENCE G/M-CFU-C GROWTH[a]

Dose ($\times 10^{-6}$ M)	Experiment I[b]	Experiment II[b]
Control	83 ± 9.5	41 ± 8
3.1	42 ± 5 (51%)	28 ± 7.5 (68%)[c]
6.2	32.7 ± 10 (39%)[d]	29.3 ± 2.5 (71.5%)
12.5	9 ± 3 (11%)[d]	22.7 ± 1.8 (55%)
25	0	15 ± 1.5 (37%)[d]

[a] Adherent stromal cell colonies were grown for 14 days and then exposed to the indicated hydroquinone dose for 3 days, after which time the medium was removed and an agar layer was plated over the adherent stromal cells and cultured for 7 days.

[b] Values represent the mean of triplicate cultures ± SE. Experiments were performed on separate days.

[c] Numbers in parentheses represent percentage of control.

[d] Significantly different from control, $p < 0.05$.

TABLE 4

EFFECT OF CATECHOL ON THE ABILITY OF ADHERENT STROMAL CELLS TO INFLUENCE G/M-CFU-C GROWTH[a]

Dose ($\times 10^{-6}$ M)	Experiment I[b]	Experiment II[b]
Control	26 ± 2	47 ± 5
25	20 ± 3 (77%)[c]	36 ± 1 (74%)
50	14 ± 2[d] (54%)	33 ± 10 (68%)
100	5 ± 1[d] (19%)	10 ± 2[d] (20%)
200	3 ± 1.5[d] (11.5%)	1 ± 0.5[d] (2%)

[a] Adherent stromal cell colonies were grown for 14 days and then exposed to the indicated catechol dose for 3 days, after which time the medium was removed and an agar layer was plated over the adherent stromal cells and cultured for 7 days.

[b] Values represent the mean of triplicate cultures ± SE. Experiments were performed on separate days.

[c] Numbers in parentheses represent percentage of control.

[d] Significantly different from control, $p < 0.05$.

TABLE 5

EFFECT OF BENZENETRIOL ON THE ABILITY OF ADHERENT STROMAL CELLS TO INFLUENCE G/M-CFU-C GROWTH[a]

Dose ($\times 10^{-6}$ M)	Experiment I[b]	Experiment II[b]
Control	39 ± 5	74.7 ± 12
3.1	35 ± 10 (90%)[c]	78.7 ± 7 (100%)
25	42 ± 5 (108%)	66 ± 6 (88%)
100	19 ± 4[d] (48%)	30.3 ± 2[d] (41%)
500	2.3 ± 1[d] (5.9%)	1 ± 1[d] (1.34%)

[a] Adherent stromal cell colonies were grown for 14 days and then exposed to the indicated benzenetriol dose for 3 days, after which time the medium was removed and an agar layer was plated over the adherent stromal cells and cultured for 7 more days.

[b] Values represent the mean of triplicate cultures ± SE. Experiments were performed on separate days.

[c] Numbers in parentheses represent percentage of control.

[d] Significantly different from control, $p < 0.05$.

in each experiment produced 55 to 127 colonies per dish, in comparison with 26 to 83 colonies per dish in control cultures with adherent cells only. Although the data are not presented, CSF controls were always included in each coculture experiment as an indication that G/M colonies were capable of growing.

DISCUSSION

Benzene metabolite toxicity to bone marrow cells *in vitro* was first demonstrated by Harrison and Randall (1948). They observed that mM concentrations of hydroquinone, catechol, and phenol inhibited bone marrow granulocyte growth in culture. In contrast, benzene itself did not inhibit granulocyte growth with any concentration tested in their studies. More recent evidence indicates that benzene cytotoxicity to the bone marrow is due to its metabolism to more reactive metabolites including phenol, hydroquinone, and catechol (Tunek *et al.*, 1981; Irons *et al.*, 1982; Rickert *et al.*, 1981; Snyder *et al.*, 1982). Within the bone marrow benzene has been shown to be selectively toxic toward hemopoietic stem cells and progenitor cells of intermediate maturity (Uyeki *et al.*, 1977; Irons *et al.*, 1979; Tunek *et al.*, 1981). Our research extends these observations of selective toxicity of benzene to the bone marrow by demonstrating that benzene metabolites are harmful to mouse bone marrow stromal cells *in vitro*. Exposure to benzene metabolites decreased stromal cell survivability and proliferation as measured by adherent cell colony formation and led to a decrease in stromal cell supported hemopoiesis.

Bone marrow stromal cells, a major component of the hemopoietic microenvironment *in vivo,* form adherent colonies consisting of fibroblasts and macrophages *in vitro* (Bentley, 1982; Zipori and Bol, 1979; Werts *et al.*, 1980). A compound which is toxic to the stromal cells *in vitro* will decrease the number of stromal cell adherent colonies compared to control values. All the benzene metabolites tested were toxic to the stromal cells and caused a decrease in adherent colony number (Figs. 1 to 5). The order of toxicity, based on inhibition of colony formation to 50% of control level (TD50), was hydroquinone, benzoquinone, benzenetriol, catechol, and phenol (Table 1). The TD50 for each benzene metabolite was determined by probit analysis as a means of comparing the relative toxicities of each metabolite. Due to the high concentrations required to induce toxicity, the TD50s for benzenetriol, catechol, and phenol are only projected values, so that while benzenetriol may have a higher projected TD50, graphically, catechol appears to be more toxic (Figs. 3 and 4).

Although some dose–response curves within replicate experiments for different metabolites showed variability due to the nature of the cultures, our results are supported by several studies which demonstrate similar relative benzene metabolite toxicities toward hemopoietic cells *in vitro,* in cell culture medium plus serum. Hydroquinone and benzoquinone in culture with mouse lymphocytes will inhibit mitogenesis at 10^{-6} *M*. Catechol and benzenetriol inhibit mouse lymphocyte mitogenesis at 10^{-5} M, while phenol is not toxic at concentrations less than 10^{-3} M (Irons *et al.*, 1982; Pfeiffer and Irons, 1981). In addition, Morimoto and Wolff (1980) demonstrated similar relative metabolite toxicities measuring mitosis of human lymphocytes in culture. Hydroquinone and catechol inhibited mitosis at doses of 4×10^{-5} M, while phenol was toxic at a dose of 10^{-3} M. Together with our present results these studies provide strong evidence that, *in vitro,* hydroquinone and benzoquinone are essentially equitoxic to stromal cells whereas catechol, benzentriol, and phenol, respectively, are considerably less toxic.

Experiments by Harigaya *et al.* (1981) and Garnett *et al.* (1983) also lend support to the notion that bone marrow stromal cells are adversely affected by benzene and its metabolites. Harigaya *et al.* (1981) demonstrated that inhalation of benzene by mice will dam-

age hemopoietic stem cells and will also injure bone marrow stromal cells. Harigaya used a liquid bone marrow culture system developed by Dexter *et al.* (1977) in which the adherent stromal cells supported and maintained hemopoietic stem cells derived from the spleen (CFU-S). The bone marrow stromal cells from the benzene exposed mice did not support hemopoietic stem cell growth for as long as control cultures were able to support stem cell growth. Garnett *et al.* (1983) using conditions similar to these reported by Harigaya *et al.* (1981) demonstrated an alteration in bone marrow stromal cell populations developed *in vitro* from benzene exposed mice as compared to control mice. Garnett *et al.* (1983) demonstrated a decrease in acid-phosphatase positive cells in the cultures of benzene exposed mice suggesting an alteration in the metabolism of stromal cells. Our study extends these results by demonstrating direct toxicity of benzene metabolites to the bone marrow stromal cells *in vitro.* Furthermore, while Harigaya *et al.* (1981) demonstrated that bone marrow from benzene exposed mice will not support spleen derived hemopoietic stem cells, our results indicate that bone marrow stromal cells exposed to benzene metabolites show a dose-related decrease in ability to induce granulocyte/monocyte hemopoiesis.

During hemopoiesis bone marrow stromal cells of the hemopoietic microenvironment interact with the maturing hemopoietic cells and may regulate blood cell growth and differentiation (Zipori *et al.,* 1982; Dexter, 1982; Bentley, 1982; Lambertsen and Weiss, 1983). The stromal cell, precursor cell coculture system provides an *in vitro* model of the hemopoietic microenvironment (Bentley, 1982; Zipori, 1981; Dexter, 1983; Werts *et al.* 1980). In our coculture system (based on the method of Zipori, 1981) the adherent stromal cell layer will induce hemopoietic precursor cells to form granulocyte/monocyte colonies. Exposure of the adherent stromal cell layer to benzene metabolites decreased granulocyte/monocyte colony formation. Doses between 1 to 10^{-6} M hydroquinone or benzoquinone inhibited granulocyte/monocyte colony formation by 50% when compared to control values. Catechol and benzenetriol required doses of 50 to 100 $\times$ 10^{-6} M to cause a 50% decrease in colony formation (Tables 2 to 5).

Reduced granulocyte/monocyte colony formation in coculture with addition of metabolite corresponded with decreased adherent stromal cell colony numbers (data not shown). Such a relationship between stromal colony numbers and granulocyte/monocyte formation after exposure to metabolite *in vitro* suggests that the decrease in granulocyte/monocyte colony formation is due to decreases in total stromal cell number and probably not due to functional changes among the supporting stromal cells. However, in a recent study in our laboratory (data not shown) in which we administered benzene ip to mice, we found that acute *in vivo* exposure to benzene led to increased stromal cell colony formation *in vitro* when compared with cultures from control mice. Even with an increase in stromal colony formation the efficiency of these stromal cells to induce granulocyte/monocyte colony formation in coculture was reduced below control values. Thus, *in vivo* exposure to benzene or its metabolite can apparently alter stromal cells supported hemopoiesis (function). Our results stress the importance of the bone marrow stromal cell in the regulation of hemopoiesis. Further studies are necessary to understand the relationship between benzene exposure *in vivo* and benzene exposure *in vitro* and toxicity to the bone marrow stroma.

Differences observed in the relative toxicities of the benzene metabolites toward stromal cells may be related to differences in cellular metabolism or oxidation. Graham *et al.* (1978) noted that toxicity of polyphenols may be due to autooxidation of the compound to highly reactive free radical products or due to the sulfhydryl group reactivity of the polyphenols. Benzene can undergo oxidation via a multistep process to hydroqui-

none and catechol in the liver and to a slight extent in the bone marrow (Andrews *et al.*, 1979; Irons *et al.*, 1979; Johansson and Ingelman-Sundberg, 1983). Hydroquinone and catechol are thought to be formed mainly in the liver and then to accumulate in the bone marrow where they may induce toxicity. Current evidence suggests that hydroquinone is further oxidized to benzoquinone by a microsomal enzyme, perhaps peroxidase (Lunte and Kissinger, 1983; Sawahata and Neal, 1983). Benzoquinone may form covalent binding with macromolecules which can inactivate enzyme systems and disrupt the cells internal environment (Irons and Neptun, 1980; Tunek *et al.*, 1980). Catechol may also undergo enzymatic oxidation to a benzoquinone without free radical formation (Mason, 1979; Irons *et al.*, 1982). The proposed differences in metabolism of these metabolites may account in part for the significant differences in toxicity to the stromal cells *in vitro*. Another important factor which must be considered is the differences in reactivity of these metabolites with protein within the medium which would decrease the amount of free metabolite available to effect the stromal cells. Benzoquinone is especially reactive and may have an extremely short half life in the presence of serum proteins, which may account for the increased cellular toxicity of benzoquinone as compared to hydroquinone when lymphocytes are pretreated with these metabolites in the absence of serum proteins (Irons *et al.*, 1982). Further research to examine the importance of metabolic pathways and metabolite reactivity is needed to better understand the mechanisms of metabolite toxicity in the bone marrow.

In summary, our results demonstrate that benzene metabolites are toxic to mouse bone marrow stromal cells *in vitro*. A comparison of the relative toxicities of the major metabolites indicated that hydroquinone and benzoquinone were the most toxic metabolites. Toxicity to the stromal cells resulted in decreased stromal cell colonies and a reduction of stromal cell induced granulocyte/monocyte colony formation. Research in our laboratory (unpublished data) and by Garnett *et al.* (1983) and Harigaya *et al.* (1981) has demonstrated that benzene administered *in vivo* will influence bone marrow stromal cells and may alter stromal cell: hemopoietic cell interactions. These results point out the importance of stromal cells in hemopoietic regulation and that injury to the bone marrow stroma may be a significant factor in benzene-induced myelotoxicity.

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