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Suppression of Bone Marrow Stromal Cell Function by Benzene and Hydroquinone Is Ameliorated by Indomethacin

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Suppression of Bone Marrow Stromal Cell Function by Benzene and Hydroquinone Is Ameliorated by Indomethacin. GAIDO, K. W., AND WIERDA, D. (1987). *Toxicol. Appl. Pharmacol.* **89**, 378–390. Administration of benzene to mice will inhibit bone marrow stromal cell-supported hemopoiesis in culture. Hydroquinone, a major metabolite of benzene, will cause a similar inhibition of stromal cell function *in vitro*. Stromal cells produce both an inducer (colony-stimulating factor) and an inhibitor (prostaglandin E_2; PGE_2) of hemopoiesis. This research was conducted to determine if prostaglandin synthesis is involved in the suppression of stromal cell function by benzene and hydroquinone. Male B6C3F1 mice were administered benzene (100 mg/kg), indomethacin (1 mg/kg), or benzene plus indomethacin twice a day for 4 consecutive days. On Day 5 bone marrow cells were removed to determine the effect of treatment. In a second series of experiments mouse bone marrow stromal cells in culture were treated with hydroquinone (10^{-7} to 10^{-4} M), indomethacin (10^{-6} M), or a combination of hydroquinone plus indomethacin. Stromal cell function was based on the ability of the treated stromal cells to support granulocyte/monocyte colony development in coculture. The results demonstrated that preadministration of indomethacin *in vivo* ameliorated benzene-induced inhibition of bone marrow stromal cell function. *In vitro*, indomethacin ameliorated hydroquinone toxicity to stromal cell function. Benzene administration *in vivo* induced elevated PGE_2 in bone marrow samples which were prevented by preadministration of indomethacin. However, hydroquinone *in vitro* did not induce a consistent increase in PGE_2 levels. These results suggested that toxicity to stromal cells was not due solely to increased prostaglandin synthetase activity.

Benzene is a widely used yet highly toxic chemical compound. Exposure to benzene can result in agranulocytosis, aplastic anemia, and myelogenous leukemia (Laskin and Goldstein, 1977; Cohen *et al.*, 1978; Snyder *et al.*, 1981). Benzene metabolism is important for the expression of toxicity (Irons *et al.*, 1982; Goldstein *et al.*, 1982). Both inhibition (Andrews *et al.*, 1979; Rickert *et al.*, 1981) and induction (Greenlee and Irons, 1981; Wierda *et al.*, 1981) of benzene metabolism will result in a decrease in benzene toxicity, suggesting that an intermediate metabolite is responsible for the toxic effects associated with benzene exposure. Phenol, hydroquinone, and catechol are major products of benzene metabolism (Rickert *et al.*, 1981; Longacre *et al.*, 1981; Greenlee and Irons, 1981; Tunek and Oesch, 1982). At least two of these metabolites, catechol and hydroquinone, concentrate within the bone marrow (Rickert *et al.*, 1981; Greenlee *et al.*, 1981) where they can interact with hemopoietic precursor cells. Bolcsak and Nerland (1983) demonstrated that *in vivo* administration of either phenol, hydroquinone, or catechol to mice will significantly inhibit erythropoiesis. Administration of hydroquinone or catechol to mice will also produce a reduction in functional B lymphocytes (Wierda and Irons,

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1982). *In vitro,* hydroquinone and catechol will inhibit lymphocyte activation (Irons *et al.,* 1982), induce sister chromatid exchange (Morimoto and Wolf, 1980), and inhibit mRNA synthesis (Post *et al.,* 1984). Aside from demonstrating that phenol, hydroquinone, and catechol are relatively toxic to hemopoietic cells, these studies suggest that one mechanism of benzene toxicity is via direct interaction of benzene metabolites with hemopoietic cells.

A second possible mechanism of benzene toxicity is an action of benzene, or its metabolites, on bone marrow stromal cells. Bone marrow stromal cells act as an essential component for hemopoiesis by forming a supporting matrix for developing precursor cells and releasing soluble factors involved in the regulation of hemopoiesis (Lichtman, 1981; Zipori, 1981; Allen and Dexter, 1984; Wilson, 1984). Previous studies have demonstrated that administration of benzene or phenol *in vivo* (Harigaya *et al.,* 1981; Gaido and Wierda, 1985; Garnett *et al.,* 1983) will alter cellular characteristics of the bone marrow stromal cells and inhibit the ability of bone marrow stromal cells to support hemopoiesis in culture. In addition *in vitro* exposure of bone marrow stromal cells to phenol, catechol, or hydroquinone will produce similar results (Gaido and Wierda, 1984), further demonstrating a role for metabolite formation in benzene-induced hemotoxicity.

Bone marrow stromal cells can influence myelopoiesis in several ways. Stromal cell produce colony-stimulating factors (CSF), growth factors, which induce myelopoietic cell proliferation, differentiation, and maturation (Metcalf, 1984; Wilson, 1984). Extracellular matrix components produced by bone marrow stromal cells may also regulate myelopoiesis (Zuckerman and Rhodes, 1985; Dexter *et al.,* 1985; Spooncer *et al.,* 1983). Prostaglandin E_2 (PGE_2), produced and released by bone marrow stromal cells, can act as a negative regulator of myelopoiesis. *In vivo* administration of PGE_2 can block the ability of myelopoietic cells to respond to colony-stimulating factor (Gentile and Pelus, 1987) and can inhibit colony-stimulating factor production by peritoneal macrophages and bone marrow stromal cells (Kurland *et al.,* 1978; Pelus *et al.,* 1981; Kriegler *et al.,* 1984). In culture, colony-stimulating factor can induce peritoneal macrophages or bone marrow stromal cells to produce PGE_2 (Kurland, 1978; Moore *et al.,* 1985; Schlick *et al.,* 1984). As the concentration of PGE_2 increases in culture it can inhibit further CSF production by bone marrow stromal cells and therefore down regulate myelopoiesis. Thus, bone marrow stromal cells, by producing both an inducer (CSF) and an inhibitor (PGE_2), can act to regulate myelopoiesis via a feedback control system (Kurland and Moore, 1977; Dayer *et al.,* 1985; Rich and Kubanek, 1985).

Phenol and hydroquinone may influence prostaglandin production under certain conditions. Hydroquinone induces prostaglandin biosynthesis in methylcholanthrene-transformed mouse BALB/3T3 fibroblasts (Polsky-Cynkin *et al.,* 1976). Phenol inhibits prostaglandin formation by bone marrow microsomes (Gollmer *et al.,* 1984). In purified enzyme systems phenol or hydroquinone can stimulate or inhibit prostaglandin biosynthesis in a concentration-dependent fashion (Hemler and Lands, 1980). One possible mechanism by which these agents alter prostaglandin synthesis may be via cooxidation with prostaglandin synthetase (Mason and Chignell, 1982; Hemler and Lands, 1980; Ross *et al.,* 1985). Through cooxidation, phenol and hydroquinone can be activated to more reactive intermediates, such as semiquinone, which bind to cellular macromolecules and inhibit cellular enzymes (Tunek *et al.,* 1982; Irons and Sawahata, 1985). The tendency of phenolic metabolites to cooxidize with prostaglandin synthetase and alter PGE_2 synthesis may contribute to benzene hematotoxicity (Das, 1978, 1979; Gollmer *et al.,* 1984; Smart and Zannoni, 1984). The goal of this study was to determine whether toxicity to stromal cell function induced by

benzene *in vivo* or hydroquinone *in vitro* could be prevented with indomethacin, an inhibitor of the cyclooxygenase component of prostaglandin synthetase. Second, we examined whether alterations in stromal cell function induced by hydroquinone exposure *in vitro* were reflected by changes in culture PGE_2 levels.

METHODS

Mice. Male B6C3F1 mice were obtained from Jackson Labs (Bar Harbour, ME). Mice were 6 to 12 weeks old when used in our study and kept four to a cage with hardwood bedding, had free access to feed (Wayne certified Lab Blox; Allied Mills, Inc., Chicago, IL) and tap water, and were housed in the West Virginia University Vivarium which is under the supervision of a full-time veterinarian. A 12-hr light cycle (6:00 AM to 6:00 PM) and an average temperature of 75 ± 5°F were maintained.

Chemicals. Hydroquinone (Sigma Chemical Co., St. Louis, MO) was diluted to indicated concentrations in sterile saline (Travenol, Chicago, IL). PGE_2 and indomethacin (Sigma) were prepared as stock solutions of 5×10^{-2} M in 95% ethanol and further diluted to the indicated concentrations in sterile saline or phosphate-buffered saline. The concentration of ethanol in culture never exceeded 0.5%. Preliminary studies indicated that this concentration of ethanol had no effect on the bone marrow stromal cell cultures. All solutions were prepared immediately before addition to culture.

Dosing interval. Benzene (Burdick & Jackson Laboratories, Inc., Muskegon, MI), indomethacin, or a combination of indomethacin plus benzene was administered ip to mice, twice a day at a 5- to 7-hr interval, for 4 consecutive days. This procedure was based on previous experiments which showed that a divided dosing schedule for benzene was sufficient to cause myelotoxicity (Gaido and Wierda, 1985). Benzene was given as a neat solution using a microliter syringe at a dose of 100 mg/kg body wt. Indomethacin was dissolved as described above and given at a dose of 1 mg/kg body wt. This dose of indomethacin was chosen in order to remain below the dose of indomethacin which inhibits cytochrome *P*-450 metabolism (5 mg/kg) (Burke *et al.*, 1983). Mice treated with benzene plus indomethacin were given indomethacin 1 hr prior to benzene administration. In this manner, mice received 200 mg/kg benzene, 2 mg/kg indomethacin, or 200 mg/kg benzene plus 2 mg/kg indomethacin per day. The amount of ethanol (used to dissolve indomethacin) administered to mice receiving indomethacin was 42 mg/kg. A decision was made to exclude ethanol controls based on a preliminary experiment which indicated that 95 mg/kg of ethanol, administered 2×/day for 4 days, exerted no effect on bone marrow cell number or stromal cell-dependent myelopoiesis. Each experiment consisted of an untreated control group, an indomethacin-treated group, a benzene-treated group, and a benzene plus indomethacin-treated group with two mice for each group. These experiments were conducted on four separate occasions over a period of 3 months.

Bone marrow cell suspensions. Mice were killed by cervical dislocation and both femurs and tibias from each mouse were removed. The bone marrow cells from individual mice were flushed from the marrow cavities into 5 ml of medium in 60-mm petri dishes (Falcon 1007, Fisher Scientific, Pittsburgh, PA). Medium consisted of RPMI 1640 supplemented with 7.5% horse serum and 7.5% fetal bovine serum (Hyclone Labs, Logan, UT) plus 2 μM glutamine, 0.02 mg/ml of gentamycin, and 100 μg/ml of sodium pyruvate. Marrow plugs were aspirated several times with a Pasteur pipet to suspend the cells. Cell number was determined by counting nucleated cells with a hemacytometer.

Mouse bone marrow adherent stromal cell cultures. Adherent stromal cell cultures were established as previously described (Gaido and Wierda, 1984). Marrow cell suspensions (2×10^6 cells/ml) in RPMI medium were placed into 35-mm plastic petri dishes (2 ml/dish) (Falcon 3046). When indicated, the appropriate concentrations of hydroquinone, PGE_2, or indomethacin were also added. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 and air. On Day 3 of culture, the medium and nonadherent cells were removed and the cultures were reconstituted with 2 ml of fresh media plus the appropriate drug concentration. The stromal cell cultures were terminated on Day 7 and stained with Wright–Giemsa stain. Adherent stromal cells form discrete colonies of fibroblasts and macrophages, with macrophages scattered between colonies. To assess growth in these cultures, colony number was determined by viewing the culture plates with an inverted microscope.

Mouse bone marrow coculture with adherent stromal cell layer. Cocultures were established as previously described (Gaido and Wierda, 1984). Bone marrow cells were plated as described above for adherent stromal cell cultures and the adherent stromal layer was allowed to develop for 7 days. On Day 7, the appropriate concentrations of either hydroquinone, indomethacin, or PGE_2 were added to the cultures and incubated for another 3 days. On Day 10, all media and drug were removed and fresh nontreated bone marrow cells (2×10^5 cells/ml) in 0.5 ml of RPMI medium plus a 0.5-ml mixture of 2× RPMI:0.6% Bacto agar were layered over the adherent stromal layer. The 2× RPMI was supplemented with 15% fetal calf serum, 15% horse serum, 2 μg/ml DEAE-dextran, 11.2 mM glutamine, and 0.1 mM 2-mercaptoethanol. The cocultures were incubated for an additional 7 days after which time the number of granulocyte/monocyte colonies which developed in the agar layer was determined and compared with control values.

Granulocyte/monocyte colony-forming unit cell assay (G/M-CFU-C). Bone marrow cells were plated (2×10^5 cells/ml) in 0.5 ml of RPMI medium plus a 0.5-ml mixture of 2× RPMI:0.6% Bacto agar. Each culture was supplemented with 15% L929-conditioned medium, a source of CSF. The cultures were incubated for 7 days after which time the number of granulocyte/monocyte colonies which developed in agar were determined and compared with control values.

PGE_2 radioimmunoassay. A radioimmunoassay (RIA) was used to determine PGE_2 levels in culture and bone marrow cell suspensions. A fractional precipitation technique was used to separate bound PGE_2 from unbound PGE_2 (Chard, 1982). Samples to be assayed (0.1 ml) were added to siliconized borosilicate test tubes containing 0.1 ml of ^3H-labeled PGE_2 (NEN Research Products, MA). Duplicate tubes were used for each sample. Rabbit anti-PGE–BSA (0.1 ml; Seragen Inc., MA) was added to each tube. The samples were allowed to equilibrate for 2 hr at room temperature and then 1 ml of 20% polyethylene glycol was added to each tube. The tubes were mixed with a vortex mixer and the solutions were allowed to precipitate overnight at 4°C. After sitting overnight the tubes were spun in a centrifuge at 2000*g* for 30 min at 4°C. The supernatant was then aspirated from each tube and the pellet remaining within each tube was resuspended with 500 μl of RIA buffer. The RIA buffer consisted of 0.9% saline plus 0.1% bovine γ-globulin, 0.01 M Na_2HPO_4, and 0.1% NaN_3. After resuspension each sample was added into 5 ml of Scintiverse II (Fischer Scientific Co., NJ) and the activity was determined using the ^3H setting on a Packard scintillation counter. A standard curve was prepared with each assay. Standard PGE_2 (Sigma) solutions were prepared in RPMI culture medium to account for any interference by serum lipids. Serum supplement that was used with the RPMI culture medium for determining a standard curve was previously charcoal adsorbed to remove PGE_2 (Chard, 1982).

Statistics. Statistics and computations were done with computer programs by Tallarida and Murray (1981). Statistical significance for all experiments was determined at the 5% level of significance. Significant treatment effects were determined by one-way analysis of variance. Comparisons between treatment groups and control values were analyzed by Dunnett's *t* test.

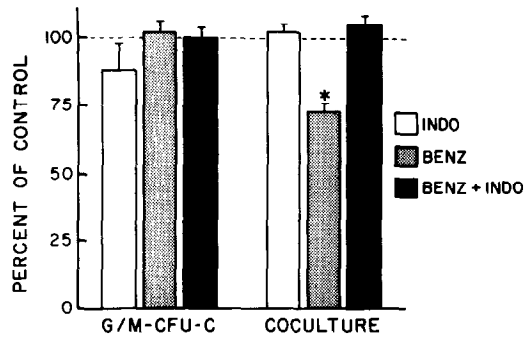

FIG. 1. The effect of benzene and indomethacin administration on granulocyte/monocyte colony formation in agar (G/M-CFU-C) and on stromal cell-supported granulocyte/monocyte colony formation in coculture. For G/M-CFU-C assays, marrow cells from control or treated mice were plated in agar with 15% L929-conditioned medium. For cocultures, nontreated bone marrow cells were plated in agar over adherent stromal cells established from control or treated mice. In both assays the number of granulocyte/monocyte colonies formed in the agar layer was determined after 7 days. Each point represents the mean response per mouse expressed as percentage of untreated control ±SE calculated from four separate experiments with two mice in each group in each experiment. Control cultures averaged 94 ± 3 G/M colonies in the G/M-CFU-C assay and 100 ± 3 G/M colonies in the coculture assay. *Values significantly different from control ($p < 0.05$) as determined with Dunnett's *t* test.

RESULTS

The Effect of Benzene and Indomethacin Administration in Vivo on Stromal Cell Function in Vitro

To determine if benzene toxicity was due in part to prostaglandin synthesis *in vivo*, mice were administered either benzene (100 mg/kg, 2×/day for 4 days), indomethacin (1 mg/kg, 2×/day for 4 days), or a combination of benzene plus indomethacin (indomethacin was administered 1 hr prior to benzene injection). On Day 5 bone marrow cells were removed and used in culture. Two separate assays were performed: the first assay (G/M-CFU-C) was used to determine the effect of benzene and indomethacin on the ability of myelopoietic cells to survive and proliferate in culture. The second assay (coculture) was used to determine if benzene and indomethacin affected the ability of bone marrow stromal cells to support granulocyte/monocyte colony formation. As shown in Fig. 1, neither indomethacin, benzene, nor a combination of benzene plus indomethacin had a statistically significant effect on G/M-CFU-C numbers as compared with control cultures. In

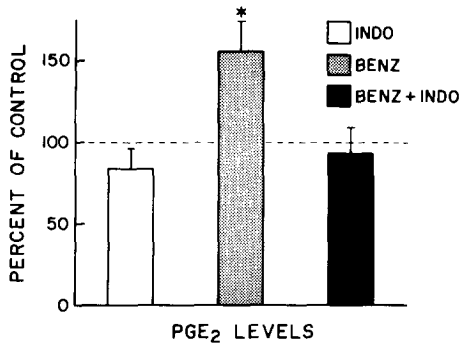

FIG. 2. Effect of benzene and indomethacin administration on PGE_2 levels in bone marrow samples. Bone marrow from two tibias plus two femurs was flushed into 0.5 ml media. PGE_2 levels were determined using a radioimmunoassay. Each point represents the mean value expressed as percentage of control ±SE calculated from three mice for each treatment group and six mice in the untreated group. Control values averaged 3.55 ± 0.3 mg PGE_2 per sample. *Values significantly different from control ($p < 0.05$) as determined with Dunnett's t test.

contrast, benzene significantly inhibited the ability of the stromal cells to support granulocyte/monocyte colony formation in coculture by 27%. Pretreatment of the mice with indomethacin 1 hr prior to treatment with benzene during the dosing regimen protected against benzene-induced stromal cell toxicity. Indomethacin did not have a significant effect on bone marrow stromal cell-supported granulocyte/monocyte colony formation.

Determination of Bone Marrow PGE_2 Levels

Bone marrow samples from benzene-treated mice were obtained on Day 5 of treatment by flushing femurs and tibias of individual mice with 0.5 ml of RPMI medium and assayed for PGE_2 concentrations (Fig. 2). Control PGE_2 concentrations from six mice averaged 3.55 ± 0.3 ng PGE_2 per bone marrow sample. Indomethacin had no significant effect on PGE_2 concentration. In contrast, benzene increased PGE_2 in bone marrow samples by 56% above control. Concentrations of PGE_2 remained at control levels in mice treated with indomethacin plus benzene. Thus, indomethacin at a dose which ameliorated toxicity to bone marrow stroma also prevented a benzene-induced increase in bone marrow PGE_2.

Effect of PGE_2 and Indomethacin on Stromal Cell Function

To determine whether PGE_2 influenced stromal cell-supported hemopoiesis in our coculture system, mouse bone marrow stromal cells were incubated in the presence of either 10^{-10} to 10^{-16} M PGE_2 or 10^{-8} to 10^{-4} M indomethacin (Fig. 3). Indomethacin or PGE_2 was added to adherent stromal cell cultures on Day 7 and incubated for another 3 days before fresh nonexposed bone marrow cells in agar were plated over the adherent stromal layer. Preliminary experiments indi-

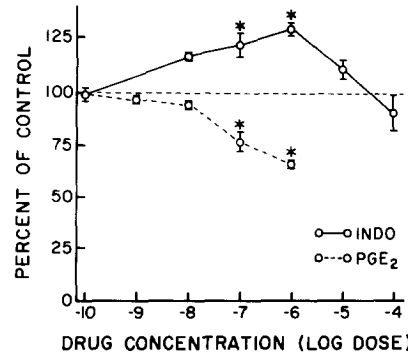

FIG. 3. Effect of indomethacin and PGE_2 treatment on the ability of bone marrow stromal cells to support granulocyte/monocyte colony formation in coculture. Indomethacin or PGE_2 was added to adherent bone marrow cell cultures on Day 7 of incubation and the cultures were further incubated for 3 days. On Day 10 nonexposed bone marrow cells in agar were plated over the adherent layer and the number of granulocyte/monocyte colonies which developed in the agar layer after 7 days incubation was determined. Each point represents the mean number of granulocyte/monocyte colonies expressed as percentage of control values ±SE calculated from four separate experiments with duplicate cultures for each dose. Control values averaged 181 ± 5 colonies per culture. *Values significantly different from control ($p < 0.05$) as determined with Dunnett's t test.

cated that within the concentration ranges listed above, neither PGE_2 nor indomethacin had a significant effect on total numbers of adherent stromal cells (data not shown). This was determined by the removal of the adherent stromal cells from culture on Day 10 in a separate series of companion cultures that were not used for coculture. The adherent stromal cells in these companion cultures were removed with trypsin and cell number per culture was determined using a hemacytometer. While these agents did not affect total adherent bone marrow stromal cell number, both indomethacin and PGE_2 had a significant effect on stromal cell function. As shown in Fig. 3, PGE_2 significantly inhibited stromal cell-supported granulocyte/monocyte colony formation by 23% at 10^{-7} M and by 34% at 10^{-6} M. Indomethacin significantly enhanced stromal cell-supported granulocyte/monocyte colony formation by 22% at 10^{-7} M and by 31% at 10^{-6} M.

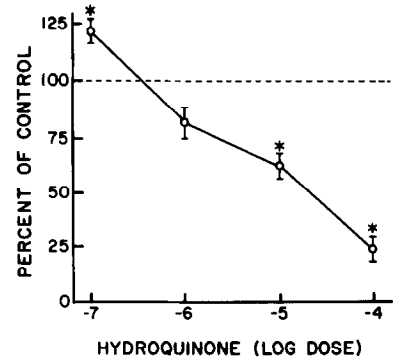

FIG. 4. Effect of hydroquinone on the ability of bone marrow stromal cells to support granulocyte/monocyte colony formation in coculture. Culture conditions were similar to those described in Fig. 2. Each point represents the mean number of granulocyte/monocyte colonies per culture expressed as percentage of control ±SE calculated from four separate experiments with duplicate cultures for each dose. Control cultures averaged 136 ± 4 G/M colonies per culture. *Values significantly different from control ($p < 0.05$) as determined with Dunnett's t test.

Effect of Hydroquinone on Stromal Cell Function

Previous results (Gaido and Wierda, 1984) indicated that, of the primary metabolites of benzene, hydroquinone was most toxic to bone marrow stromal cell function. To characterize further the effect of hydroquinone on stromal cell function, mouse bone marrow stromal cells were exposed to hydroquinone (10^{-7} to 10^{-4} M) on Day 7 of culture and incubated for an additional 3 days to determine the effect of hydroquinone on bone marrow stromal cell function in culture. On Day 10 of culture the medium was removed and fresh nonexposed bone marrow cells in agar were plated over the adherent stromal layer. Hydroquinone produced a dose-dependent decrease in stromal cell-supported granulocyte/monocyte colony formation (Fig. 4). Granulocyte/monocyte colony formation was decreased by 39% at 10^{-5} M and by 77% at 10^{-4} M hydroquinone. In contrast, stromal cell-supported granulocyte-monocyte formation was enhanced by 23% at 10^{-7} M. In a separate series of experiments (data not shown) it was determined that concentrations of hydroquinone from 10^{-7} to 10^{-5} M added on Day 7 of culture had no effect on total adherent stromal cell number by Day 10 of culture, although cell number was decreased by 39% with 10^{-4} M hydroquinone. These results demonstrated that hydroquinone at concentrations which did not alter total stromal cell number significantly altered stromal cell-supported hemopoiesis.

Effect of Indomethacin Pretreatment on Hydroquinone-Induced Alterations in Bone Marrow Stromal Cell Function

To determine whether hydroquinone affected stromal cell function by interaction with prostaglandin synthesis in mouse stromal cells, adherent cultures were treated as described above, except that some cultures were treated with indomethacin (10^{-6} M) 1 hr prior to treatment with hydroquinone (Fig.

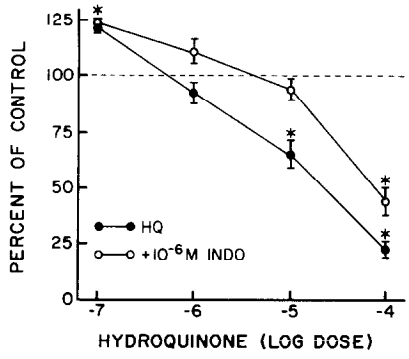

FIG. 5. The effect of hydroquinone plus indomethacin on the ability of bone marrow stromal cells to support granulocyte/monocyte colony formation in coculture. Culture conditions were similar to those described in Fig. 2 except that cultures were treated with indomethacin plus hydroquinone (indomethacin was added 1 hr prior to hydroquinone addition). Each point represents the mean number of granulocyte/monocyte colonies per culture expressed as percentage of control ±SE calculated from five separate experiments with duplicate cultures for each dose. *Values significantly different from control values ($p < 0.05$) as determined with Dunnett's t test. Slopes for each dose–response curve were determined to be significantly different by linear regression analysis in conjunction with a paired t test. In addition, individual treatment groups were compared (hydroquinone vs indomethacin) by a paired t test and all values were significantly different with the exception of treatment groups exposed to 10^{-7} M hydroquinone.

5). Similar to the results presented earlier (Fig. 4), hydroquinone exposure produced a dose-dependent decrease in stromal cell-supported granulocyte/monocyte colony formation. Pretreatment of adherent cells with indomethacin significantly ameliorated hydroquinone-induced inhibition of stromal cell function. While hydroquinone alone at 10^{-5} M inhibited stromal cell-supported granulocyte/monocyte colony formation by 36%, pretreatment of the adherent cells with 10^{-6} M indomethacin allowed stromal cells exposed to 10^{-5} M hydroquinone to support granulocyte/monocyte colony formation to 93% of the control level. Stromal cells did not demonstrate a further enhancement of function when 10^{-6} M indomethacin was added with 10^{-7} M hydroquinone.

Determination of PGE_2 Levels in Hydroquinone- and Indomethacin-Treated Cultures

Medium was removed from the adherent stromal cell cultures described above and assayed for PGE_2 levels by radioimmunoassay (Fig. 6). Lipopolysaccharide (LPS) was used as a positive control to induce PGE_2 production which averaged 620 ± 80 pg/culture and indomethacin pretreatment reduced LPS-induced PGE_2 to 211 ± 33 pg/culture (data not shown). The addition of increasing concentrations of hydroquinone tended to elevate detectable PGE_2 concentrations; however, this trend was not statistically significant. The prior addition (1 hr before hydroquinone) of 10^{-6} M indomethacin to cultures treated with hydroquinone did not result in a significant reduction in PGE_2 concentration.

DISCUSSION

Metabolism of benzene is important in the expression of myelotoxicity. Phenol is a primary metabolite of benzene (Irons *et al.*, 1982; Tunek *et al.*, 1980; Wallin *et al.*, 1985) and can be produced from benzene within

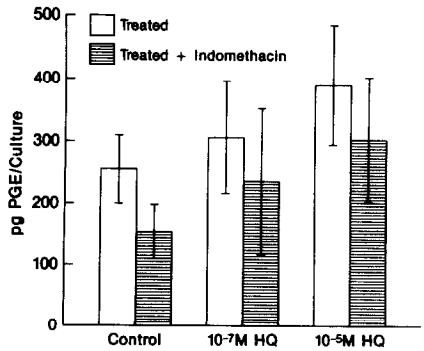

FIG. 6. Induction of PGE_2 synthesis in adherent stromal cell cultures exposed to hydroquinone. Hydroquinone or indomethacin was added on Day 7 of culture. On Day 10, medium was removed and assayed for PGE_2 by RIA. Each bar represents the average amount of PGE_2 detected ±SE in pooled duplicate cultures from six separate experiments. Control cultures averaged 252 ± 60 pg PGE_2 per milliliter.

the bone marrow (Irons *et al.*, 1980). Two major metabolites of phenol, hydroquinone and catechol, concentrate within the marrow after exposure to benzene (Greenlee *et al.*, 1981; Rickert *et al.*, 1981). Hydroquinone can be subsequently metabolized to semiquinone and benzoquinone which are reactive with cellular macromolecules and thereby alter enzyme function and disrupt cell processes (Irons and Sawahata, 1985; Wallin *et al.*, 1985; Tunek and Oesch, 1982). Lunte and Kissinger (1983) confirmed that benzene is metabolized to phenol and then to hydroquinone by cytochrome *P*-450. Lunte and Kissinger (1983) further suggested that the enzymatic conversion of hydroquinone to a reactive binding species may not involve cytochrome *P*-450 activity. Studies by Smart and Zannoni (1984) and Sawahata and Neal (1983) have demonstrated a role for bone marrow peroxidase enzymes in the conversion of both phenol and hydroquinone to reactive metabolites. Prostaglandin synthetase contains a peroxidase component and is present in almost all cells of the body including bone marrow stromal cells. In addition, prostaglandin synthetase can cooxidase phenols and hydroquinones (Marnett *et al.*, 1975; Mason and Chignell, 1982; Hemler and Lands, 1980). During cooxidation, hydroquinone theoretically is converted to a semiquinone radical capable of binding to cellular macromolecules and disrupting cellular processes (Irons and Sawahata, 1985; Moldeus *et al.*, 1985).

The results of our study implicate prostaglandin synthetase activity in the expression of benzene toxicity. *In vivo* administration of indomethacin, an inhibitor of the cyclooxygenase component of prostaglandin synthetase, ameliorated benzene toxicity toward bone marrow stromal cells (Fig. 1). The dose of benzene used in our study (100 mg/kg, 2×/day, 4 days) was based on previous work in our lab (Gaido and Wierda, 1985) which indicated a preferential toxicity of benzene toward bone marrow stromal cells without significant changes in mouse weight, bone marrow cell number, or granulocyte/monocyte precursor number. Indomethacin protected against benzene toxicity at a dose (1 mg/kg, 2×/day, 4 days) which had no effect on mouse weight, bone marrow cell number (data not shown), or granulocyte/monocyte precursor number (Fig. 1). In addition, indomethacin prevented the increase of PGE_2 levels induced by benzene (Fig. 2). The dose of indomethacin used in these experiments was based on studies by Burke *et al.* (1983) which demonstrated that no alterations in microsomal *P*-450 metabolism occurred in rats given less than 5 mg/kg of indomethacin for 4 days. This consideration was necessary to minimize the potential induction of *P*-450 metabolism of benzene by indomethacin. At this time we cannot completely rule out an effect of indomethacin on cellular *P*-450 metabolism or on other metabolic pathways. However, the dose of indomethacin necessary to alter other enzyme systems usually is much higher than the dose necessary to inhibit prostaglandin synthetase activity (Burke *et al.*, 1983; Fontagne *et al.*, 1983; Kitchen *et al.*, 1985). In the present study, prostaglandin synthesis was never completely inhibited, suggesting that the dose of indomethacin was well below that necessary for significantly altering other enzymatic pathways. The 2 mg/kg dose of indomethacin used in our study was also below the 6 mg/kg/day level which induced enhanced myelopoiesis in mice (Fontagne *et al.*, 1983).

Work by Wolf and Trentin (1968) first indicated a role for bone marrow stromal cells in myelopoiesis. Since then it has been determined that stromal cells release many soluble mediators which enhance myelopoiesis including colony-stimulating factors and interleukin 1 (Metcalf, 1984; Moore *et al.*, 1985; Rich and Kubanek, 1985; Zipori, 1981). An inhibitory factor that can be produced in high concentrations by bone marrow cells is PGE_2 (Kurland *et al.*, 1978; Moore *et al.*, 1985; Schlick *et al.*, 1984). In culture, high levels of colony-stimulating factor will induce PGE_2 production by both peritoneal macrophages

(Kurland, 1978) and bone marrow stromal cells (Moore *et al.*, 1985). High levels of PGE_2, in turn, reportedly inhibit further colony-stimulating factor production by these cells (Kurland, 1978). Kurland and Moore (1977) suggested that colony-stimulating factor and PGE_2 constitute a feedback control system which regulates myelopoiesis within the bone marrow. Pelus *et al.* (1981) demonstrated that PGE_2 inhibition is qualitative in nature. Lower levels of PGE_2 (10^{-10} to 10^{-8} M) preferentially inhibit monocytopoiesis, while higher levels (10^{-8} to 10^{-6} M) inhibit both monocytopoiesis and granulocytopoiesis. In addition, Moore *et al.* (1985) demonstrated that complete inhibition of PGE_2 production by bone marrow cells with indomethacin enhanced monocyte colony formation by only 20% which suggested the presence of other inhibitory factors produced by bone marrow cells. We have confirmed these studies in our lab by demonstrating that high levels of PGE_2 significantly inhibited bone marrow stromal cell-supported myelopoiesis (Fig. 3). The addition of 10^{-7} M PGE_2 to bone marrow stromal cell cultures reduced granulocyte/monocyte colony formation by 23%. The preferential effect of PGE_2 on monocytopoiesis versus granulocytopoiesis was not determined and must still be considered with lower doses of PGE_2. In contrast, indomethacin enhanced granulocyte/monocyte colony formation by 23% at 10^{-7} M and by 31% at 10^{-6} M. Our values agree with the 20% enhancement of monocyte colony formation induced by indomethacin in the study by Moore *et al.* (1985). These results demonstrated an ability of PGE_2 to suppress stromal-dependent myelopoiesis, but the high levels of PGE_2 required to produce a significant reduction in granulocyte/monocyte colony formation, and the inability of indomethacin to enhance granulocyte/monocyte colony formation greater than 30% above control levels, indicated that in addition to PGE_2 production, other events were involved in the inhibition of myelopoiesis. One of these events could include the production of other inhibitory factors by stromal cells. Lactoferrin and interferon-γ are examples of soluble mediators implicated in the inhibition of stromal cell function (Pelus *et al.*, 1981; Moore *et al.*, 1985). We did not test for these inhibitors in the present study.

Das (1978, 1979) suggested that the myelotoxicity associated with many agents may be due to their ability to alter prostaglandin synthesis. Hemler and Lands (1980) demonstrated that phenols can either enhance or inhibit prostaglandin biosynthesis in purified enzyme systems, depending on the concentration of phenol. Phenol, hydroquinone, and other phenolic compounds can alter prostaglandin synthesis via cooxidation with prostaglandin synthetase (Mason and Chignell, 1982; Hirafuji and Ogura, 1985). In adherent bone marrow stromal cell cultures, macrophages are potentially an abundant source of prostaglandin synthetase. Hydroquinone caused a dose-dependent inhibition of stromal cell-supported granulocyte/monocyte colony formation (Fig. 4). Thus, we examined whether inhibition of stromal cell function by hydroquinone may be due in whole, or in part, to cooxidation with prostaglandin synthetase (Fig. 6). PGE_2 levels in stromal cell cultures, assessed by a radioimmunoassay procedure, were used as an indirect indicator of prostaglandin synthetase activity. Lipopolysaccharide stimulation of macrophages was used as a positive control to induce prostaglandin synthetase activity and to raise PGE_2 levels in culture (Kurland, 1978), whereas indomethacin was used to reduce prostaglandin synthetase activity and lower PGE_2 levels in culture. The addition of indomethacin to adherent stromal cell cultures slightly reduced PGE_2 levels in cultures exposed to hydroquinone (Fig. 6) but significantly ameliorated hydroquinone toxicity to stromal-dependent myelopoiesis (Fig. 5).

As shown in Fig. 6, levels of PGE_2 in our cultures were comparable to those determined in other studies (Kurland, 1978; Kurland *et al.*, 1978; Schlick *et al.*, 1984). In contrast to *in vivo* benzene administration, the

addition of 10^{-7} or 10^{-5} M hydroquinone to bone marrow stromal cell cultures failed to induce a consistent increase in PGE_2 concentrations, nor did indomethacin significantly reduce PGE_2 concentrations within these cultures. The range of PGE_2 concentrations detected was $0.43–1.1 \times 10^{-9}$ M PGE_2. Pelus *et al.* (1981) demonstrated the $10^{-10}–10^{-8}$ M PGE_2 inhibited monocytopoiesis when added directly to agar that contained G/M-CFU-C precursors and exogenous colony-stimulating factors. This differs, however, from our system in that we pretreated established stromal cells for 3 days with PGE_2, washed out any remaining PGE_2, and then bioassayed for stromal-dependent myelopoiesis in our coculture procedure. Under these conditions, 10^{-7} M concentrations of PGE_2 were necessary to inhibit significantly stromal-dependent myelopoiesis, and adding increasing concentrations of hydroquinone did not induce PGE_2 production to inhibitory levels. We interpreted these results as indicating that other toxic events occurred in stromal cells exposed to hydroquinone. One possibility may include metabolic activation of hydroquinone to a semiquinone or benzoquinone, a process which can be mediated through cooxidation by the peroxidase component of prostaglandin synthetase (Marnett and Eling, 1983; Irons and Sawahata, 1985). However, no published studies are yet available which address the quantity of prostaglandin synthetase in bone marrow tissue.

Indomethacin has been reported to exert other actions on cells in addition to its primary effect on cyclooxygenase activity which could account for its apparent protective effect in these studies. Indomethacin given orally to mice (6 mg/kg) for 4 days increases cyclic adenosine 3′:5′-monophosphate and guanosine 3′:5′-monophosphate amounts in bone marrow concomitant with increased myelopoiesis (Fontagne *et al.*, 1983). Indomethacin and other nonsteroidal anti-inflammatory drugs also augment the release of leukotrienes by shunting substrate arachidonate from prostaglandins to the 5-lipoxygenase pathway (Kuehl *et al.*, 1984). At least two leukotrienes, LTC4 and LTD4, have been shown to participate as positive modulators of mouse and human G/M-CFU-C formation *in vitro* (Ziboh *et al.*, 1986). Macrophages can produce significant amounts of leukotrienes, and these cells are an integral part of the stromal milieu. Thus, we cannot exclude the possibility that these or other unknown mechanisms may be responsible for the protective effect of indomethacin on stromal cell function observed in these studies.

In summary, we have demonstrated that preadministration of indomethacin *in vivo* ameliorated benzene-induced inhibition of bone marrow stromal cell function and that indomethacin *in vitro* ameliorated hydroquinone toxicity to stromal cell function. Benzene administration *in vivo* induced elevated PGE_2 levels in bone marrow samples which were prevented by indomethacin. However, hydroquinone *in vitro* did not induce a significant increase in PGE_2 levels. We suggest that toxicity to stromal cells is the result of further activation of benzene or hydroquinone to other toxic moieties within the stroma and that amelioration of toxicity by indomethacin may be attributed to other potential biological activities of the drug in addition to inhibition of prostaglandin synthetase.

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