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Cell-Specific Metabolism in Mouse Bone Marrow Stroma: Studies of Activation and Detoxification of Benzene Metabolites

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SUMMARY

Two of the major cell types in bone marrow stroma, macrophages and fibroblasts, have been shown to be important regulators of both myelopoiesis and lymphopoiesis. The enzymology relating to cell-specific metabolism of phenolic metabolites of benzene in isolated mouse bone marrow stromal cells was examined. Fibroblastoid stromal cells had elevated glutathione-*S*-transferase (4.5-fold) and DT-diaphorase (4-fold) activity relative to macrophages, whereas macrophages demonstrated increased UDP-glucuronosyltransferase (UDP-GT, 7.5-fold) and peroxidase activity relative to stromal fibroblasts. UDP-GT and glutathione-*S*-transferase activities in macrophages and fibroblasts, respectively, were significantly greater than those in unpurified white marrow. Aryl sulfotransferase activity could not be detected in either bone marrow-derived macrophages or fibroblasts, and there were no significant differences in GSH content between the two cell types. Because UDP-GT activity is high in macrophages, these data suggest that DT-diaphorase levels would be rate limiting in the detoxification of benzene-derived quinones in bone marrow macrophages. The peroxidase responsible for bioactivation of benzene-derived phenolic metabolites in bone marrow macrophages is unknown but has been suggested to be prostaglandin H synthase (PGS). Hydrogen peroxide, but not arachidonic acid, supported metabolism of hydroquinone to reactive species in bone marrow-derived macrophage lysates. These data do not support a major role for PGS in peroxidase-mediated bioactivation of hydroquinone in bone marrow-derived macrophages, although PGS mRNA could be detected in these cells. Similarly, hydrogen peroxide, but not arachidonic acid, supported metabolism of hydroquinone in a human bone marrow homogenate. Peroxidase-mediated interactions between phenolic metabolites of benzene occurred in bone marrow-derived macrophages. Bioactivation of hydroquinone to species that would bind to acid-insoluble cellular macromolecules was increased by phenol and was markedly stimulated by catechol. Bioactivation of catechol was also stimulated by phenol but was inhibited by hydroquinone. These data define the enzymology and the cell-specific metabolism of benzene metabolites in bone marrow stroma and demonstrate that interactions between phenolic metabolites may contribute to the toxicity of benzene in this critical bone marrow compartment.

Benzene is a widespread industrial pollutant that induces bone marrow toxicity and leukemia after chronic exposure (1, 2). The precise mechanisms underlying benzene-induced myelotoxicity and leukemia are unclear, but there is considerable evidence to show that benzene requires metabolic activation in order to exert its adverse effects (3–6). The major hepatic metabolites of benzene are phenol, catechol, and hydroquinone (7, 8). Catechol and hydroquinone have been shown to persist in bone marrow after benzene exposure (9). The bone marrow is rich in peroxidase activity (10), and phenolic metabolites of benzene can be activated by peroxidases to reactive quinone derivatives (11–14). The metabolism of benzene is complex, however, and other potentially reactive intermediates such as benzene oxide, *trans,trans*-muconaldehyde, and 1,2,4-benzenetriol may also play a role in benzene-induced toxicity (4, 8).

Although the major site of metabolism of benzene is the liver, benzene toxicity is invoked primarily at the level of the bone marrow (1, 2). Within the marrow both hematopoietic progenitor cells and bone marrow stromal cells are potential targets of benzene toxicity. The stroma provides a supporting framework within the medullary cavity of the bone marrow for the process of blood cell development (15, 16). Stromal cells regulate the growth and differentiation of both myeloid and lymphoid progenitors and are critical in the regulation of normal hemopoiesis (16). Stromal cells are intimately associated with developing blood cells and regulate hemopoiesis via direct cell to cell interactions, the production of extracellular matrix components, and the secretion of soluble mediators such as cyto-

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ABBREVIATIONS: UDP-GT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography; 2-ME, 2-mercaptoethanol; HBSS, Hanks' balanced salt solution; PGS, prostaglandin H synthase; PAPS, adenosine 3′-phosphate 5′-phosphosulfate.

kines and eicosanoids (16–18). Stromal cells have been implicated as a potential target of benzene toxicity after administration of benzene to mice (19) or after treatment of stromal cell cultures with benzene and its metabolites *in vitro* (20, 21). A recent study has shown a potent and prolonged effect of administration of benzene via inhalation to mice on subsequent bone marrow stromal cell function *in vitro* (22). The adherent cell layer isolated from mice after benzene administration had a diminished capacity to support hemopoiesis and the cellularity of adherent layers was greatly reduced.

Studies of the metabolic capability of bone marrow in general and stroma in particular are scarce. Such studies would have applicability not only to studies of mechanisms of action of environmental myelotoxins but also to therapeutic agents, because the dose-limiting toxicity of many clinically used alkylating agents is myeloid in nature. Bone marrow stroma consists of at least four different cell types (i.e., adipocytes, endothelial cells, macrophages, and fibroblasts). Two of these cell types, macrophages and fibroblastoid stromal cells, are known to be particularly important in the regulation of hemopoiesis within the marrow (16, 23, 24). Macrophages rather than fibroblastoid cells were shown to be selectively sensitive to the toxic effects of the benzene metabolite hydroquinone (24). In previous work, we presented evidence that suggested that this cellular selectivity could be explained on the basis of cell-specific metabolism within bone marrow stroma (25). Specifically, the bone marrow-derived macrophage had an increased ability to activate hydroquinone via peroxidatic oxidation to the reactive 1,4-benzoquinone and a decreased ability to detoxify the quinone product via the two-electron quinone reductase DT-diaphorase (EC 1.6.99.2.). This work demonstrates the utility of examining cell-specific metabolism in bone marrow and is in accord with previous work (26), which showed that the peroxidase/DT-diaphorase ratio was a major determinant of the covalent binding of [^{14}C]phenol in guinea pig hepatic microsomes. The critical role of DT-diaphorase in the protection of bone marrow stroma against reactive quinones such as 1,4-benzoquinone has recently been independently confirmed (27, 28).

In view of the potential importance of bone marrow stroma in hemopoiesis and our limited knowledge of the metabolic capability of this critical compartment, we have extended our work to characterize more completely the enzymology contributing to metabolic activation and deactivation in bone marrow-derived macrophages and fibroblastoid stromal cells. We have examined the levels of GSH and the activities of phase II metabolic pathways that are critical in the deactivation of benzene-derived phenolic metabolites and quinones formed by further oxidation of these phenolics. Specifically, the activities of UDP-GT (EC 2.4.1.17), PAPS-dependent arylsulfotransferase (EC 2.8.2.1), and glutathione-*S*-transferase (EC 2.5.1.18) were examined in unpurified white bone marrow, bone marrow-derived macrophages, and stromal fibroblasts. Peroxidases are pivotal in the bioactivation of phenolics in bone marrow (5, 12), but the identity of the peroxidase(s) responsible for activation in bone marrow-derived macrophages is unclear. In addition, peroxidase-mediated interactions between phenolic metabolites of benzene have been proposed to be important in benzene toxicity (29), but whether such interactions occur in bone marrow stroma is unknown. In an attempt to address these questions, we have examined the mechanisms of peroxidatic bioactivation of benzene-derived phenolic metabolites, both alone and in combination, in purified murine bone marrow stromal cell populations.

Materials and Methods

Chemicals. [^{14}C]Hydroquinone (22.2 mCi/mmol) was purchased from Wizard Labs. (Davis, CA), and [^{14}C]catechol (7.4 mCi/mmol) was obtained from Sigma Chemical Co. (St. Louis, MO). Arachidonic acid was purchased from Nu-Check-Prep Inc. (Elysain, MN). RPMI 1640 medium and HBSS were obtained from JRH Biosciences (Lenexa, KS). L-Glutamine, gentamicin, and HEPES were purchased from BRL/GIBCO (Gaithersburg, MD) and fetal bovine serum was obtained from Hyclone (Logan, UT). All other chemicals were from Sigma.

Animals. Male B6C3F$_1$ mice (6–8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were kept for at least 1 week in an animal care facility before use.

Isolation of mouse bone marrow. Mice were sacrificed by cervical dislocation and the femurs and tibias were removed. The marrow was flushed from the bones using a 25-gauge needle, into phosphate-buffered saline. Cells were pelleted and subjected to two hypotonic lysis steps to remove mature red cells (30). The cells were pelleted once more and resuspended in phosphate-buffered saline. These cells are referred to as white bone marrow. The cells were then lysed using a probe sonicator at 0–4° for subsequent determination of enzyme activities and GSH content.

Isolation of bone marrow-derived macrophages. Mice were sacrificed and marrow was removed as described above but marrow was pooled in collecting medium. Collecting medium consisted of RPMI 1640 with glutamine supplemented with 10 mM HEPES, 5×10^{-5} M 2-ME, 0.04 mg/ml gentamicin, and 10% heat-inactivated fetal bovine serum. The marrow plugs were gently dispersed by aspiration using a 25-gauge needle. Macrophages were isolated from bone marrow cell suspensions based on the method of Tushinski *et al.* (31), modified as previously described (24). Briefly, cells were cultured in RPMI 1640 medium supplemented with L929 conditioned medium. L929 conditioned medium is a potent source of macrophage colony-stimulating factor and stimulates proliferation and maturation of macrophages (31). Nonadherent cells were transferred to new tissue culture plates on days 1 and 3. Adherent cells on day 5 were used as macrophages. Day 5 cultures were stained for nonspecific esterase activity using α-naphthyl acetate (Sigma kit 91-A) and >95% of cells stained positive.

Culture of a long term fibroblastoid cell line. A fibroblastoid cell line, designated LTF, was obtained as described (24). Briefly, single-cell clones were obtained from a long term mouse bone marrow culture by isolation of single cells using glass rings. The cells were maintained in the same medium used for macrophage culture but without L929 conditioned medium.

Human bone marrow. Human bone marrow was obtained at autopsy (65-year-old man; death due to cardiopulmonary failure) and a predominantly white cell fraction was obtained by repeated hypotonic lysis. The final cell fraction was homogenized in 0.1 M potassium phosphate buffer, pH 7.4, using a Teflon pestle at 0–4°. Subsequent incubations with hydroquinone were performed at 37°.

Covalent binding of radiolabeled substrates to acid-insoluble macromolecules. Adherent macrophage and LTF cells were washed twice with HBSS (pH 7.4, containing calcium and magnesium), removed by scraping, and sonicated using a Branson model S125 sonifier. In some experiments cells were used intact. Cell disruption was confirmed by microscopy. Aliquots of cell lysates were incubated at 37° in HBSS, pH 7.4, in a shaking water bath. The purity of [^{14}C]hydroquinone and [^{14}C]catechol was verified by HPLC analysis (11, 25) before use. Incubations (1 ml) were initiated by the addition of either arachidonic acid or hydrogen peroxide and were terminated by the addition of 70% TCA (0.1 ml/incubation). Control incubations were performed in the absence of arachidonic acid or hydrogen peroxide. In some experiments, incubations were performed in the presence of hydrogen

peroxide or arachidonic acid but using lysates that had been subjected to prior boiling. This treatment reduced levels of covalent binding to less than those observed in the absence of hydrogen peroxide or arachidonate. The TCA-insoluble material was precipitated and washed with TCA, 70% ethanol, and ethanol/ether (1:1), as described previously (25). The air-dried precipitate was solubilized in 0.5 ml of 1 N sodium hydroxide and then neutralized with 1 M HCl before addition of Scintiverse II scintillation cocktail (Fisher Scientific). Radioactivity covalently bound to acid-insoluble macromolecules was determined in a Packard scintillation counter. Protein concentration was determined by the method of Lowry *et al.*, and data were expressed as nanomoles of [^{14}C]hydroquinone or [^{14}C]catechol equivalents bound per milligram of protein.

GSH and enzyme assays. GSH was determined by HPLC using the method of Reed *et al.* (32). Glutathione-*S*-transferase activity was determined in cell lysates according to the method of Habig *et al.* (33). Peroxidase activity was determined using tetramethylbenzidine as substrate, as modified from previously described biochemical and immunoassay methods (34, 35). Briefly, aliquots of cell lysates (0.1 ml) were mixed with tetramethylbenzidine (0.1 ml of 10 mM). The tetramethylbenzidine was dissolved in water containing 0.01 ml of Tween 20/10 ml of solution. The reaction was performed in 50 mM potassium phosphate buffer (pH 6) and initiated by the addition of 0.1 ml of 10 mM hydrogen peroxide. After 4 min, the reaction was stopped with 0.1 ml of 5 M phosphoric acid. The blue chromophore (A_{max} = 650 nm) is converted to a yellow chromophore (A_{max} = 450 nm) after the addition of acid and this also increases the intensity of absorbance of the product. The absorbance values at 450 nm were read immediately after the reaction was stopped. Calibration curves were performed, using either horseradish peroxidase or human myeloperoxidase, in each experiment. Two lysate protein concentrations were assayed for each sample to ensure linearity of response, and the A_{max} at 450 nm was verified in each biological sample. This assay was more sensitive than other peroxidase assays we have employed utilizing pyrogallol or guaiacol. Using human myeloperoxidase, the detection limit was 1×10^{-5} units/ml and the calibration curve was linear ($r = 0.9994$) to 1.4×10^{-3} units/ml.

UDP-GT and aryl sulfotransferase activities were determined in bone marrow cell lysates prepared in 10 mM Tris·HCl (pH 7.0) containing 250 mM sucrose and 3 mM 2-ME. As positive controls, UDP-GT and aryl sulfotransferase activities were measured in hepatic microsomes and cytosol, respectively, prepared from male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) as described previously (36). Rat liver microsomes were isolated from a 33% homogenate in 50 mM Tris·HCl, pH 7.0, containing 150 mM potassium chloride (37). Hepatic cytosol was prepared from a 25% homogenate in 10 mM Tris·HCl, pH 7.0, containing 250 mM sucrose and 3 mM 2-ME (38). Incubation conditions for the measurement of UDP-GT activity were as described previously (37). The final incubation volume was 0.5 ml and contained 200 mM Tris·HCl, pH 7.7, 10 mM magnesium chloride, 1.25 mM D-saccharic-1,4-lactone, 4 mM UDPGA, 1 mM α-naphthol, and 0.5–1.0 mg/ml lysate or microsomal protein. Reactions were terminated by the addition of 3 volumes of TCA/glycine (0.5 M TCA and 0.8 M glycine, mixed 1:1) (39) and the samples were stored at −70° until analysis by HPLC. Aryl sulfotransferase activity was measured in 1-ml incubates containing 250 mM potassium phosphate buffer, pH 7.4, 5 mM 2-ME, 0.25 mM PAPS, 1 mM β-naphthol, and 0.5–1.0 mg/ml lysate or cytosolic protein (38, 40). Reactions were terminated by the addition of 80 μl of 2 M acetic acid to 300 μl of the reaction mixture (38), and the samples were stored at −70° until analysis by HPLC. Protein concentrations were determined by the method of Lowry *et al.* (41).

Formation of the glucuronic acid and sulfate conjugates of α-naphthol and β-naphthol, respectively, was measured by modification of the reverse phase, ion-pair HPLC method described by Rhodes and Houston (42), using an Econosil C_{18} column (5 μm, 4.6 × 250 mm; Alltech Associates, Deerfield, IL) with UV absorbance detection at 280 nm. Analysis of β-naphthol sulfate was conducted with an isocratic system eluted with a mobile phase composed of 92% solvent A (35% acetonitrile containing 0.2 mM tetrabutylammonium hydrogen sulfate) and 8% solvent B (100% acetonitrile) at a flow rate of 1 ml/min. Analysis of α-naphthol glucuronide was conducted with a gradient mobile phase system beginning with 100% solvent A for 1 min and proceeding linearly to 92% solvent A over 6 min at 1 ml/min. Retention times of α-naphthol, α-naphthol glucuronide, α-naphthol sulfate, β-naphthol, and β-naphthol sulfate were identified by co-chromatography with standards purchased from Sigma. Quantitation of α-naphthol glucuronide and β-naphthol sulfate formation was based upon linear regression curves generated by HPLC analysis of the commercially available standards. HPLC analysis of hydroquinone was performed as described previously (25).

Competitive polymerase chain reaction to determine mRNA for PGS. This was determined as described previously (43).

Statistical analyses. Statistics were performed using the CRUNCH interactive statistical package. Analyses of GSH content and enzyme activity between macrophages and fibroblasts were performed using a Student *t* test with a significance level of $p < 0.05$. Comparisons of binding data were analyzed by a one-way analysis of variance followed by a Dunnett's *post hoc* test for multiple comparisons with a single control. Where multiple comparisons were made using binding data, a Newman-Keuls test was used as the *post hoc* test. A significance value of $p < 0.05$ or less was used as a test of significance. All possible statistical comparisons between groups are not represented in the figures.

Results

The peroxidase activity in bone marrow stromal macrophages was 1.85 ± 0.27 AU/min/mg of protein (mean ± standard error, five experiments), whereas it could not be detected in fibroblastoid stromal cells (detection limit, 2.5×10^{-2} AU/min/mg of protein). These data, using more sensitive assays for peroxidase activity, confirm our previous work (25).

The activities of other enzyme systems in macrophages and fibroblasts that may contribute to activation and deactivation of benzene metabolites, together with the concentration of GSH in the different cell populations, are shown in Table 1. UDP-GT activity in the bone marrow-derived macrophage was 7.5-fold higher than in the stromal fibroblast, which had activity similar to that of white marrow (Table 1). The activity of UDP-GT in the bone marrow-derived macrophage was 34% of hepatic values. This probably represents an underestimate of the true value because the hepatic values were based on microsomal protein, whereas the macrophage values were based on total cellular protein. In view of the limited quantities of purified bone marrow-derived macrophages available, it was impractical to prepare microsomes from this cell population. Aryl sulfotransferase activity was not detected in either cell type, although activity in rat liver cytosol, which was used as a positive control, was readily detectable and was consistent with literature values (Table 1). Glutathione-*S*-transferase activity was found to be significantly elevated, by 4.5- and 1.5-fold, in stromal fibroblasts relative to macrophages and white marrow, respectively. No significant differences were observed in the content of GSH in bone marrow-derived macrophages and stromal fibroblasts, although the content of both cell types was significantly higher than that of white bone marrow (Table 1).

Results from studies using peritoneal macrophages and enzyme inhibitors *in vivo* (44–46) have suggested that the peroxidase responsible for bioactivation of phenolic metabolites of benzene in bone marrow-derived macrophages is PGS. Activation of hydroquinone to reactive species capable of covalent

TABLE 1

Enzyme activities and GSH concentrations

Values represent mean ± standard error, with the number of determinations indicated in parentheses.

	GSH	GST activity[a]	UDP-GT activity	Aryl ST activity
	nmol/mg of protein	*nmol/min/mg of protein*	*nmol/min/mg of protein*	*nmol/min/mg of protein*
WBM	6.9 ± 0.7 (3)	61.20 ± 2.34 (4)	0.68 ± 0.06 (4)	ND[b]
Mϕ	14.1 ± 0.6 (6)[c]	20.10 ± 2.77 (4)[c]	5.66 ± 0.09 (4)[c]	ND
LTF	14.6 ± 0.8 (3)[c]	92.13 ± 7.45 (4)[c,d]	0.75 ± 0.04 (4)[d]	ND
Rat liver				
Microsomes			16.50 ± 0.95 (6)	
Cytosol				1.26 ± 0.16 (6)

[a] GST, glutathione *S*-transferase; Aryl ST, aryl sulfotransfersase; WBM, whole bone marrow (unpurified white bone marrow obtained after red blood cell lysis); Mϕ, bone marrow-derived macrophages; LTF, stromal fibroblasts.

[b] ND, not detected. Detection limit of HPLC assay is 0.7 μM, which corresponds to an approximate detection limit of 0.03 nmol/min/mg of protein under the present experimental conditions.

[c] Significantly different from whole bone marrow value, $p < 0.01$.

[d] Significantly different from bone marrow-derived macrophage value, $p < 0.01$.

binding to protein or alkylation of thiols by peritoneal macrophage lysates was dependent on the presence of hydrogen peroxide, showing the peroxidatic nature of the bioactivation process (44). Covalent binding of [^{14}C]hydroquinone equivalents could also be stimulated by addition of arachidonic acid to peritoneal macrophages (44). In bone marrow-derived macrophages, we were able to demonstrate marked peroxidase-mediated bioactivation using hydrogen peroxide as a substrate (Fig. 1), confirming our previous data (25). Significant arachidonic acid-mediated stimulation of the covalent binding of [^{14}C] hydroquinone to acid-insoluble macromolecules using bone marrow-derived macrophages, however, could not be demonstrated using either lysates (Fig. 1) or whole cells (data not shown). When lysates of peritoneal macrophages were used, prepared essentially as described previously (44), we were able to demonstrate increases in both hydrogen peroxide- and arachidonic acid-dependent covalent binding of [^{14}C]hydroquinone to protein (data not shown). The level of mRNA for PGS was examined using competitive polymerase chain reaction and was found to be 2.9 amol/μg of RNA in bone marrow-derived macrophages and 0.34 amol/μg of RNA in stromal fibroblasts (mean of two separate determinations). Although these data show the presence of PGS message in bone marrow-derived macrophages, the inability of arachidonic acid to catalyze metabolism of hydroquinone to reactive species does not support a significant role for PGS in the bioactivation of hydroquinone in this cell type. Similarly, in a human bone marrow homogenate hydrogen peroxide, but not arachidonic acid, could support metabolism of hydroquinone (Fig. 2).

The covalent binding of [^{14}C]hydroquinone to acid-insoluble macromolecules is shown in Fig. 3. Hydrogen peroxide stimulated the binding of [^{14}C]hydroquinone to acid-insoluble macromolecules. Phenol significantly enhanced hydrogen peroxide-stimulated bioactivation of [^{14}C]hydroquinone in bone marrow-derived macrophages (Fig. 3), as previously reported for whole bone marrow and for purified myeloperoxidase (14, 30, 47). Of particular interest was the marked stimulation of binding of [^{14}C]hydroquinone by catechol (Fig. 3). Hydrogen peroxide could also stimulate bioactivation of [^{14}C]catechol in bone marrow-derived macrophage lysates (Fig. 4). Covalent binding of [^{14}C]catechol to acid-insoluble macromolecules was enhanced by phenol and inhibited by hydroquinone (Fig. 4), which is consistent with our previous work using unpurified preparations of rat bone marrow (11). The covalent binding of both hydroquinone and catechol in bone marrow-derived macrophages could be inhibited by GSH (data not shown) and *N*-acetylcysteine (Fig. 5), in agreement with the sulfhydryl reactivity of 1,4- and 1,2-benzoquinone.

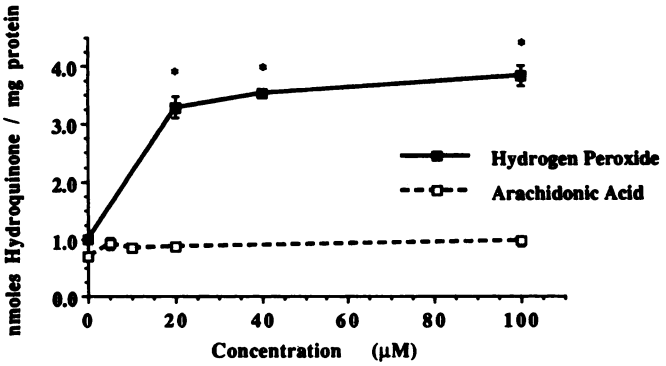

Fig. 1. Hydrogen peroxide- or arachidonic acid-dependent binding of [^{14}C]hydroquinone (0.01 mM) to acid-insoluble macromolecules in lysates of bone marrow-derived macrophages. Reactions were performed for 30 min at 37°, initiated by the addition of either hydrogen peroxide or arachidonic acid, and terminated by the addition of 0.1 ml of 70% TCA. Data represent mean ± standard error of three or four separate experiments where incubations were performed in duplicate or triplicate. *, Significantly different from control value measured in the absence of hydrogen peroxide or arachidonic acid, $p < 0.01$.

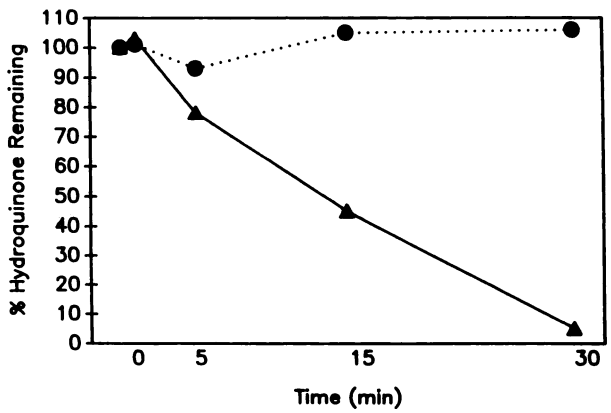

Fig. 2. Metabolism of hydroquinone by a human bone marrow homogenate. Arachidonic acid- (0.1 mM) (●) or hydrogen peroxide- (0.1 mM) (▲) dependent removal of hydroquinone (0.05 mM) was measured using a human bone marrow homogenate (13 μg of protein/ml) at 37° in potassium phosphate buffer (0.1 M), pH 7.4. Reactions were terminated by the addition of 0.1 ml of perchloric acid (70%) and were analyzed by HPLC as described.

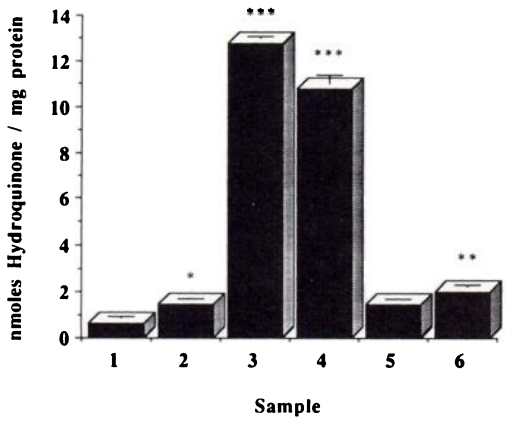

Fig. 3. Effect of catechol or phenol on the covalent binding of [^{14}C] hydroquinone (0.01 mM) to acid-insoluble macromolecules in bone marrow-derived macrophage lysates. *1*, No hydrogen peroxide; *2–6*, 0.1 mM hydrogen peroxide; *3*, plus 0.1 mM catechol; *4*, plus 1 mM catechol; *5*, plus 0.1 mM phenol; *6*, plus 1 mM phenol. Data represent mean ± standard error of three separate determinations. *, Significantly different from control without peroxide (*1*) $p < 0.01$; **, significantly different from 0.1 mM hydrogen peroxide (*2*), $p < 0.05$; ***, significantly different from 0.1 mM hydrogen peroxide (*2*), $p < 0.01$.

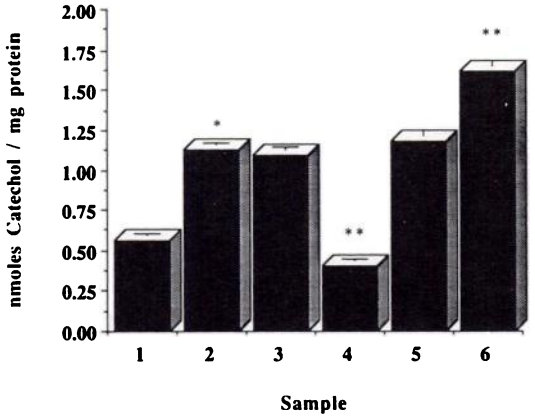

Fig. 4. Effect of hydroquinone or phenol on the covalent binding of [^{14}C] catechol (0.01 mM) to acid-insoluble macromolecules in bone marrow-derived macrophage lysates. *1*, No hydrogen peroxide; *2–6*, 0.1 mM hydrogen peroxide; *3*, plus 0.1 mM hydroquinone; *4*, plus 1 mM hydroquinone; *5*, plus 0.1 mM phenol; *6*, plus 1 mM phenol. Data represent mean ± standard error of three separate determinations. *, Significantly different from control without peroxide (*1*), $p < 0.01$; **, significantly different from 0.1 mM hydrogen peroxide (*2*), $p < 0.01$.

Discussion

Although peroxidase and DT-diaphorase are important determinants of the bone marrow toxicity of benzene metabolites, other enzyme systems are also critical to the detoxification of phenolics and their quinoid oxidation products. Phenolic compounds are particularly susceptible to UDP-GT- and aryl sulfotransferase-mediated conjugation with glucuronic acid and sulfate, respectively. Conjugation reactions would also be expected to be important to DT-diaphorase-mediated detoxification of quinoids in bone marrow stroma (Fig. 6), because once reduced the hydroquinone may undergo reoxidation to the quinone if it is not conjugated with either sulfate or glucuronic acid. In the present study aryl sulfotransferase activity could not be detected in either stromal cell type. UDP-GT activity, however, was markedly higher in bone marrow-derived macrophages than in fibroblastoid stromal cells or white bone marrow. Indeed, the activity of UDP-GT in bone marrow-derived macrophages was of the same order as hepatic levels. This suggests that the levels of DT-diaphorase are probably rate limiting in terms of detoxification of quinones in bone marrow-derived macrophages. Once the quinone is reduced, then conjugation should not be hindered by lack of UDP-GT activity (Fig. 6). These predictions, however, do not take into account whether benzene metabolites would behave similarly to the model substrates used for activity measurements, aspects of enzyme compartmentation (the majority of DT-diaphorase is cytosolic, whereas UDP-GT is microsomal), or cofactor availability. It is possible that the amount of UDPGA, the cofactor required for glucuronidation, may limit the glucuronide conjugation capacity of bone marrow macrophages. Bone marrow UDPGA concentrations have not been reported, but such a situation has been described in the liver and intestine (48, 49).

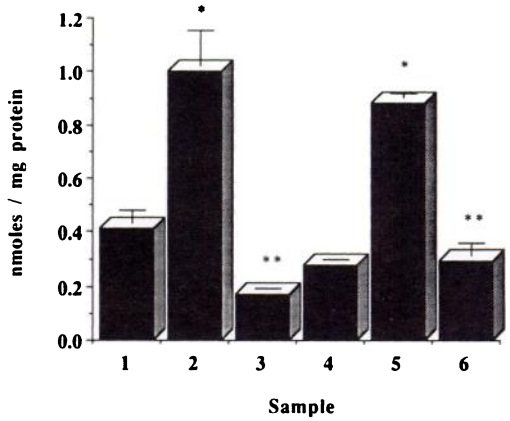

Fig. 5. Effect of *N*-acetylcysteine on covalent binding of [^{14}C]hydroquinone or [^{14}C]catechol to acid-insoluble macromolecules in bone marrow-derived macrophage lysates. Incubates *1–3* contained [^{14}C]hydroquinone (0.01 mM); incubates *4–6* contained [^{14}C]catechol (0.01 mM). Incubates *3* and *6* contained *N*-acetylcysteine (0.2 mM). *1* and *4*, no hydrogen peroxide; *2*, *3*, *5*, and *6*, 0.1 mM hydrogen peroxide. Data represent mean ± standard error of three separate determinations. *, Significantly different from control (*1* or *4*) in the absence of peroxide, $p < 0.01$; **, significantly different from incubate without *N*-acetylcysteine (*2* or *5*), $p < 0.01$.

GSH and glutathione-*S*-transferases are important in the detoxification of electrophilic species such as quinones (50). Our data show that glutathione-*S*-transferase activity is approximately 4-fold higher in fibroblastoid cells than in bone marrow-derived macrophages, whereas the content of GSH, the essential cofactor for this detoxification pathway, is not significantly different between the cell types. Glutathione-*S*-transferase activity was also enriched in the stromal fibroblast, relative to white bone marrow. This suggests that, although nonenzymatic conjugation of electrophiles with GSH would not be expected to differ between cell types, the fibroblastoid cells would show a greater propensity for enzyme-mediated, GSH conjugation reactions. Although 1,4-benzoquinone has been suggested to be a substrate for glutathione-*S*-transferase (51), there is little unequivocal evidence to suggest that glutathione-*S*-transferases can increase the rate of conjugation of 1,4-benzoquinone with glutathione. The activity of glutathione-*S*-transferases, however, may modulate the myelotoxicity of a variety of other agents such as antitumor mustards (52).

Peroxidase-mediated activation of phenolic metabolites of benzene is a major mechanism of bioactivation in bone marrow (5). Identification of the peroxidase(s) in a particular cell is

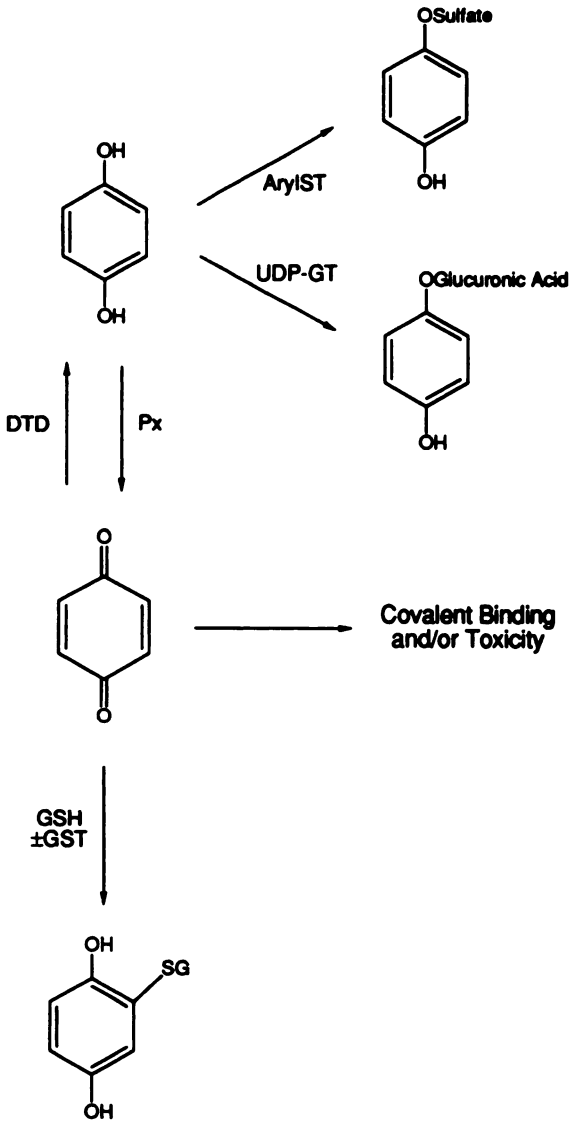

Fig. 6. Enzymology contributing to activation and detoxification of hydroquinone in bone marrow stroma. *ArylST*, aryl sulfotransferase; *DTD*, DT-diaphorase; *Px*, peroxidase; *GST*, glutathione-*S*-transferase.

complicated because of the broad substrate specificity of most peroxidases and the fact that enzyme inhibitors are not usually specific for an individual peroxidase. The peroxidase in human monocytes has been suggested to be myeloperoxidase (53), and recent studies have identified potentially novel peroxidase activities, distinct from myeloperoxidase, in alveolar macrophages (54). Schlosser *et al.* (44, 46) have implicated PGS as the peroxidase responsible for bioactivation of phenolic metabolites of benzene, based on the ability of arachidonic acid to stimulate covalent binding of [^{14}C]hydroquinone or [^{14}C]phenol to acid-insoluble macromolecules in mouse peritoneal macrophages. Both arachidonic acid and hydrogen peroxide can support PGS-mediated bioactivation of xenobiotics via co-oxidation processes (55). Metabolism and bioactivation of hydroquinone by purified PGS have been shown to be supported with equal efficiency by either hydrogen peroxide or arachidonic acid (56). We observed substantial arachidonic acid-dependent binding of [^{14}C]hydroquinone to acid-insoluble macromolecules in peritoneal macrophages but observed no increases over control levels in bone marrow-derived macrophages isolated from mice. Hydrogen peroxide stimulated covalent binding of [^{14}C]hydroquinone in bone marrow-derived macrophages, confirming the peroxidatic nature of the process. These data suggest that peritoneal macrophages are not a good model system for predicting events in bone marrow-derived macrophages. In human bone marrow the situation appears to be similar because hydrogen peroxide, but not arachidonic acid, supported metabolism of hydroquinone, although it should be stressed that this represents data from only a single autopsy sample. The level of PGS mRNA in mouse bone marrow-derived macrophages was higher than in stromal fibroblasts, although levels were considerably lower than those reported in human endothelial cells (43). Clearly, because bone marrow-derived macrophages contain PGS mRNA and synthesize prostaglandins (21), the involvement of PGS in bioactivation of phenolic metabolites cannot be discounted. The inability of arachidonic acid to support metabolism of hydroquinone, however, is not consistent with a major role for PGS in bioactivation in murine bone marrow-derived macrophages or human bone marrow and suggests that other peroxidases are primarily responsible for bioactivation.

A criticism of the hypothesis that phenolic metabolites of benzene are responsible for toxicity is that administration of the individual phenolic metabolites does not result in myelotoxicity (29, 57). Peroxidase-mediated interactions of phenolics have been known for many years (58), but the impetus for more recent benzene-related studies was the observation that coadministration of hydroquinone and phenol could reproduce the myelotoxicity associated with benzene exposure in mice (29). Phenol stimulates bioactivation of hydroquinone by purified myeloperoxidase (14, 47), in bone marrow cells *in vitro* (30), and in mouse bone marrow *in vivo* (59). We have confirmed in this study that this interaction also occurs in bone marrow-derived macrophages. The relevance of the interaction of phenol and hydroquinone to benzene-induced myelotoxicity, however, has recently been questioned on the basis of a pharmacokinetic modeling study (60). The latter study predicted higher levels of phenol and hydroquinone in bone marrow after phenol administration than after benzene administration and suggested that other metabolites must also be involved in benzene toxicity. Catechol is an attractive candidate because greater quantities would be formed from benzene, via its dihydrodiol precursor (61), than from phenol, which is metabolized primarily to hydroquinone and water-soluble conjugates (26, 62, 63). We have shown previously that catechol is bioactivated by peroxidases and by rat and human bone marrow to reactive species (11, 64). In this study, catechol was also bioactivated by bone marrow-derived macrophages and bioactivation could be stimulated by phenol and inhibited by hydroquinone. Perhaps the most striking aspect of the interactions of phenolic metabolites observed in this study was the ability of catechol to stimulate markedly the bioactivation of hydroquinone. This result may be of some significance in view of the potential involvement of catechol in benzene toxicity and is in agreement with recent work that demonstrated a synergistic genotoxic effect of catechol and hydroquinone in lymphocytes (65).

In summary, these data characterize the enzymology contributing to activation and detoxification of benzene metabolites in murine bone marrow stroma and emphasize the importance of phase II enzymes in the effective detoxification of electro-

philes. This work suggests that, although bone marrow-derived macrophages have elevated UDP-GT activity, low DT-diaphorase activity may limit the detoxification of quinones in this cell type. Our data do not support a major role for PGS in the bioactivation of phenolic metabolites of benzene in bone marrow-derived macrophages but suggest that peroxidase-mediated interactions between phenolics occurring at the level of the stroma, particularly the stimulation by catechol of hydroquinone bioactivation, may be important in benzene toxicity.

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