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Bone Marrow Stromal Cell Bioactivation and Detoxification of the Benzene Metabolite Hydroquinone: Comparison of Macrophages and Fibroblastoid Cells

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SUMMARY

Bone marrow stroma consists predominately of two cell types, macrophages and fibroblastoid stromal cells, which regulate the growth and differentiation of myelopoietic cells via the production of growth factors. We have previously shown that macrophages are more sensitive than fibroblastoid stromal cells (LTF cells) to the toxic effects of the benzene metabolite hydroquinone. In this study, the role of selective bioactivation and/or deactivation in the macrophage-selective effects of hydroquinone was examined. LTF and macrophage cultures were incubated with 10 μM [^{14}C]hydroquinone to examine differential bioactivation. After 24 hr, the amount of ^{14}C covalently bound to acid-insoluble macromolecules was determined. Macrophages had 16-fold higher levels of macromolecule-associated ^{14}C than did LTF cells. Additional experiments revealed that hydroquinone bioactivation to covalent-binding species was hydrogen peroxide dependent in macrophage homogenates. Covalent binding in companion LTF homogenates was minimal, even in the presence of excess hydrogen peroxide. These data suggest that a peroxidative event was responsible for bioactivation in macrophages and, in agreement with this, macrophages contained detectable peroxidase activity whereas LTF cells did not. Bioactivation of [^{14}C]hydroquinone to protein-binding species by peroxidase was confirmed utilizing purified human myeloperoxidase in the presence of hydrogen peroxide and ovalbumin as a protein source. High performance liquid chromatographic analysis of incubations containing purified myeloperoxidase, hydroquinone, and hydrogen peroxide showed that greater than 90% of hydroquinone was removed and could be detected stoichometrically as 1,4-benzoquinone. 1,4-Benzoquinone was confirmed as a reactive metabolite formed from hydroquinone in macrophage incubations using excess GSH and trapping the reactive quinone as its GSH conjugate, which was measured by high performance liquid chromatography with electrochemical detection. The activity of DT-diaphorase, a quinone reductase that has been invoked as a protective mechanism in quinone-induced toxicity, was 4-fold higher in LTF cells than macrophages. These data suggest that the macrophage-selective toxicity of hydroquinone results from higher levels of peroxidase-mediated bioactivation and/or lower levels of DT-diaphorase-mediated detoxification.

Benzene is a ubiquitous compound produced by both natural and industrial processes. Myelotoxicity is associated with chronic benzene exposure and is due to metabolism of benzene to reactive compounds. Benzene myelotoxicities include granulocytopenia, lymphocytopenia, thrombocytopenia, pancytopenia, aplastic anemia, and acute myelogenous leukemia (1–4). Benzene is also postulated to cause various lymphocytic leukemias (2).

Possible target cell types within the bone marrow include developing hemopoietic stem cells and stromal cells. The intimate anatomical relationship between stromal cells and hemopoietic stem cells suggests both a functional role and a structural role for the stromal cells in the differentiation and proliferation of hemopoietic cells. The stroma provides structural support for immature hemopoietic cells and produces lymphokines and monokines, such as CSF, which support bone marrow lymphopoiesis and myelopoiesis *in vitro* and *in vivo* (5–

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ABBREVIATIONS: CSF, colony-stimulating factors; MPO, myeloperoxidase; HPLC, high performance liquid chromatography; HQ-SG, 2-glutathionyl-1,4-dihydroxybenzene; PCA, perchloric acid; PBS, phosphate buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

11). In our culture system using bone marrow cells, the stroma consists of two predominant cell populations, including macrophages and large flat fibroblastoid cells (8, 12, 13). These cells interact via the production of soluble mediators to regulate lymphopoiesis and myelopoiesis. Hemopoietic stem cells rapidly die, *in vitro*, without the presence of marrow-derived adherent stromal cell monolayers, which produce CSF, or without the addition of exogenous CSF (11, 14, 15). Damage to bone marrow stromal cells by benzene exposure, therefore, could decrease the number of functional hemopoietic cells.

Benzene toxicity is associated primarily with the formation of reactive metabolites including hydroquinone, benzoquinone, phenol, and catechol. Other potentially active intermediates include 1,2,4-benzenetriol, (*trans,trans*)-muconaldehyde, 4,4′-biphenol, and 2,2-biphenol (16–22). Benzene is metabolized primarily in the liver via mixed function oxidase enzymes to phenol, hydroquinone, and catechol (22–24). Inhibition of benzene metabolism by either inhibitors of mixed function oxidase or compounds such as toluene that compete for mixed function oxidase decreases the toxicity associated with benzene exposure (25). Of the liver metabolites, hydroquinone and catechol concentrate within the bone marrow cavity (26, 27). Sabourin *et al.* (28) determined that bone marrow samples from benzene-treated mice contain significant levels of muconic acid and glucuronide and sulfate conjugates of hydroquinone, catechol, and phenol.

Metabolites of benzene generated in the liver may themselves be toxic, but recent research suggests that secondary metabolism within the bone marrow is necessary to elicit toxicity. The bone marrow contains high levels of peroxidase enzymes, such as MPO (29), which can readily oxidize phenolics to free radicals, which, via coupling or disproportiation reactions, can generate reactive quinones (30). Such reactions have been investigated utilizing both purified peroxidases and cellular systems (17, 31–34).

We have previously determined that mixed bone marrow stromal cells treated with hydroquinone had a reduced ability to support myelopoiesis (13). We examined independent populations of both macrophages and fibroblastoid cells for selective sensitivity to toxic concentrations of hydroquinone. Macrophage regulation of fibroblastoid cell CSF production, examined using reconstituted cultures, was reduced when treated with 1 μM hydroquinone. Fibroblast cell function, however, was affected only at concentrations of 100 μM. The goal of the present study was to examine the mechanism of this cell selectivity. Our results suggest that macrophages are more susceptible because of a selective ability of this cell to bioactivate hydroquinone, coupled with an inability to detoxify its reactive metabolite 1,4-benzoquinone.

Materials and Methods

Chemicals and enzymes. Hydroquinone, 1,4-benzoquinone, GSH, and hexadecyltrimethylammonium bromide were obtained from Sigma Chemical Company (St. Louis, MO). [U-^{14}C]hydroquinone was obtained from Wizard Laboratories (Davis, CA) (22.2 mCi/mmol) and American Radiolabeled Chemicals (St. Louis, MO) (25 mCi/mmol). [*glycine*-2-^{3}H]GSH (1149 mCi/mmol) was purchased from New England Nuclear Research Products, Boston, MA (DE). Human leukocyte MPO was obtained from Calbiochem (San Diego, CA).

Mice. Male B6C3F$_1$ mice, 6 to 12 weeks old when used in these studies, were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept four to a cage in microisolation cages with hardwood bedding. The mice were housed in the animal facilities at the University of Colorado School of Pharmacy or the West Virginia University Health Sciences Center. The mice had free access to feed and water and a 12-hr light-dark cycle was maintained.

Isolation and preparation of bone marrow cells. The mice were sacrificed via cervical dislocation and their femurs and tibias were removed. The marrow was flushed from the bone shaft using a 23-gauge needle and 5 ml of collecting medium. Collecting medium consisted of RPMI 1640 with glutamine (Whittaker MA Bioproducts, Walkersville, MD) supplemented with 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol (Sigma), 0.02 mg/ml gentamicin (Whittaker), and 10% heat inactivated-fetal bovine serum (Hyclone Laboratories, Logan, UT). The marrow plugs were gently aspirated using a 23-gauge needle. The suspension of single cells was counted using Turk's solution to determine total nucleated cells.

Derivation of fibroblastoid cell line. A fibroblastoid cell line, designated LTF, was obtained as previously described (13). Briefly, single-cell clones were obtained from a long term mouse bone marrow culture by isolation of single cells using glass rings (Bellco, Vineland, NJ). These cells were maintained in RPMI 1640 with glutamine containing 5×10^{-5} M 2-mercaptoethanol, 0.02 mg/ml gentamicin, and 10% fetal bovine serum.

Establishment of purified macrophage cultures. Macrophages were isolated from bone marrow cell suspensions using a modification of the procedure of Tushinski *et al.* (35), as previously described (13). Briefly, bone marrow cells were cultured in the presence of 10% L929 fibroblastoid cell-conditioned medium. L929-conditioned medium is a potent source of macrophage colony-stimulating factor and stimulates proliferation and maturation of macrophages. After a single replating of nonadherent cells, the adherent cells that had developed on day 5 were used as macrophages. Cultures consisted of >95% adherent macrophages with <5% contaminating cells.

Peroxide-dependent bioactivation of hydroquinone in cell homogenates. Adherent LTF cells and macrophages were removed by scraping and were homogenized in PBS, pH 7.4, using a motorized Teflon-glass homogenizer. One-milliliter aliquots of cell homogenate from each cell type were incubated for 30 min at 37° in a shaking water bath. Incubations consisted of cell homogenates and 10 μM [^{14}C]hydroquinone, with or without the addition of hydrogen peroxide. The purity of [^{14}C]hydroquinone was confirmed by HPLC analysis before use. Reactions were terminated and cellular macromolecules were precipitated by the addition of 0.1 ml of 60% PCA to the 1-ml samples. The acid-insoluble material was separated from the supernatant phase. The acid-insoluble fraction was exhaustively washed with successive treatments of 5% trichloroacetic acid, 70% methanol (two times), and 1:1 ethanol/ether (two times) to remove noncovalently bound radioactivity. The precipitate was solubilized in 0.5 ml of 1 N sodium hydroxide, followed by neutralization to pH 7.0 with hydrochloric acid. Equal volumes of solution were analyzed to determine levels of radioactivity and protein concentration. Radioactivity levels were determined by scintillation counting using Scintiverse (Fisher, Pittsburgh, PA) and a Packard scintillation counter. Protein concentration was determined by the method of Lowry *et al.* (36). Data are expressed as nanomol of [^{14}C]hydroquinone equivalents/mg of protein.

Examination of bioactivation of hydroquinone in macrophage and LTF cultures. Macrophage and LTF cultures were exposed to 10 μM [^{14}C]hydroquinone in serum-free medium for 24 hr at 37°. The cells were washed, removed from the tissue culture dish by scraping, collected in 3 ml of PBS, and transferred to tubes where the reaction was stopped by the addition 300 μl of 60% PCA. Radioactivity covalently bound to acid-insoluble macromolecules was then determined as described above.

Peroxidase activity in macrophage and LTF cell homogenates. Macrophage and LTF cell homogenates were prepared at a cell concentration of 1×10^8 cells/ml in 5 ml of 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer (HTAB buffer), pH 6.0 (37). The samples were subjected to freeze-thawing

three times, followed by high speed centrifugation to remove particulate material. One hundred microliters of sample were mixed with 2.9 ml of 50 mM potassium phosphate, pH 6.0, or 2.9 ml of HTAB buffer, each containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance associated with the oxidation of *o*-dianisidine was measured at 460 nm. One unit of peroxidase activity was defined as that degrading 1 μmol of peroxide/min at 25° (38). The limit of detection was 0.002 units/10^7 cells.

Examination of purified MPO-mediated bioactivation of [^{14}C] hydroquinone. Purified MPO (1 unit/ml) was assayed for its ability to convert [^{14}C]hydroquinone to a species capable of covalent binding to protein. Assays were performed using increasing concentrations of MPO. Each 1-ml assay contained 1.4–1.7 mg of ovalbumin (that had been previously heated to 100° for 1 hr and slowly cooled to minimize any endogenous oxidizing capacity); 10 μM [^{14}C]hydroquinone; 40 μM hydrogen peroxide; and 0, 0.015, 0.03, or 0.05 units of MPO as indicated for each experiment. Samples were incubated for up to 30 min in a shaking water bath at 37°. At the end of the incubation time, the reactions were stopped by the addition of 400 μl of 6% trichloroacetic acid. The samples were centrifuged to pellet the protein. The samples were exhaustively washed as described previously, solubilized in 1 M NaOH, and neutralized. Samples were analyzed for amount of covalently bound radioactivity and concentration of protein.

HPLC analysis of hydroquinone, 1,4-benzoquinone, and its GSH conjugate. HPLC analysis was performed using a Supelco 5-μm C18 reverse phase column and either UV/visible detection or electrochemical detection. All analyses were performed on a Shimadzu HPLC system equipped with a SIL6A autoinjector and a CR3A data processor.

Electrochemical detection was used for detection of the GSH conjugate of 1,4-benzoquinone, according to the method of Lunte and Kissinger (39). An isocratic elution system of 1% acetonitrile in 0.1 M acetate buffer, pH 4, was used at 1 ml/min, together with an LC-4B amperometric detector equipped with a single glassy carbon electrode. The detector potential was +0.7 V. Retention times of standard reference compounds were, for hydroquinone, 10.1 min and, for the glutathione conjugate of 1,4-benzoquinone, 19.2 min. These retention times were in excellent agreement with those reported (39). The formation of the GSH conjugate of 1,4-benzoquinone was dependent on the presence of both reactants, and the eluant corresponding to this HPLC peak contained tritium when [^{3}H]GSH was used in the reaction mixture.

UV/visible detection was used for quantification of hydroquinone and 1,4-benzoquinone and also as an alternative system for detection of the glutathione conjugate of 1,4-benzoquinone. A Supelco C18, 5-μm, reverse phase column was used, together with a linear gradient elution system of 2% solution B to 15% solution B from 2 min to 12 min. Solution B was then maintained at 15% for an additional 7 min. The flow rate was 1 ml/min. (Solution A was ammonium acetate buffer, 50 mM, pH 4, and solution B was methanol.) Detection wavelengths were chosen from spectra of the compounds (not shown) and were 288 nm for hydroquinone and 246 nm for 1,4-benzoquinone. Because reaction of 1,4-benzoquinone with GSH yields a chromophore at 304 nm (not shown), this wavelength was used for detection of the GSH conjugate. Injection volumes were 20 μl for 1,4-benzoquinone quantitation, whereas all other assays were performed using 100-μl injection volumes. Retention times using this system were, for hydroquinone, 10.3 min, for 1,4-benzoquinone, 17.3 min, and, for its glutathione conjugate, 14.7 min. Calibration curves for 1,4-benzoquinone at 246 nm and hydroquinone at 288 nm between 0 and 100 μM both had correlation coefficients of $r > 0.99$.

Incubations with purified MPO. The study of the removal of hydroquinone and appearance of 1,4-benzoquinone was performed using 0.1 mM hydroquinone, 0.2 mM H_2O_2, and 0.25 units/ml MPO in 1 ml incubations at 37° for 15 min. Reactions were stopped by addition of 0.1 ml of 60% PCA and centrifuged and the supernatant was used directly for HPLC analysis. Twenty-microliter injections were used for 1,4-benzoquinone analysis at 246 nm and 100-μl injections were used for hydroquinone analysis at 288 nm.

Analysis of GSH conjugate formation in macrophages. Incubations (1 ml) contained macrophage homogenate in PBS (0.18 mg of protein/m; cell disruption confirmed by microscopy), hydroquinone (0.01 mM), H_2O_2 (0.1 mM), and GSH (0.2 mM). Reactions were performed for 30 min in a shaking water bath at 37° and terminated by addition of 0.1 ml of 70% PCA. The samples were centrifuged and the supernatant was used directly for HPLC analysis. Under these acidic conditions the GSH conjugate of 1,4-benzoquinone was stable for at least 18 hr at room temperature. Analytical procedures using the autosampler were always completed within 8 hr, so conjugate breakdown during analysis was not a complicating factor.

Determination of DT-diaphorase activity in macrophages and LTF fibroblastoid cells. DT-diaphorase was determined essentially according to the method of Ernster (40), as modified by Benson *et al.* (41). The assay uses dicumarol-sensitive reduction of 2,6-dichlorphenolindophenol. Cells were scraped from plates and sonicated on ice in a small volume of Tris·HCl buffer (25 mM, pH 7.4). Aliquots (50–100 μl) of the samples were then used for enzyme assays. NADPH (0.2 mM)-dependent removal of 2,6-dichloroindophenol (0.04 μM) was followed at 600 nm using bovine serum albumin (0.7 mg/ml) as an activator (41). Activity inhibited by dicumarol (0.02 mM) was used as a measure of DT-diaphorase activity.

Statistical analysis. Analysis of samples was accomplished where indicated using a paired *t* test or unpaired *t* test as a test of the null hypothesis. One-way or two-way analysis of variance was performed where indicated and Dunnett's *post hoc t* test was used as a test of the null hypothesis. A level of significance of *p* equal to or less than 0.05 was used unless otherwise indicated.

Results

It was postulated that macrophages are selectively sensitive to the toxic effects of hydroquinone because of a selective ability to bioactivate hydroquinone to a species capable of binding protein and other cellular macromolecules. To examine this hypothesis, whole-cell experiments were used to investigate selective metabolism of hydroquinone. As shown in Fig. 1, macrophage cultures had 16-fold higher levels of covalently bound, [^{14}C]hydroquinone-derived radioactivity, when compared with LTF cells.

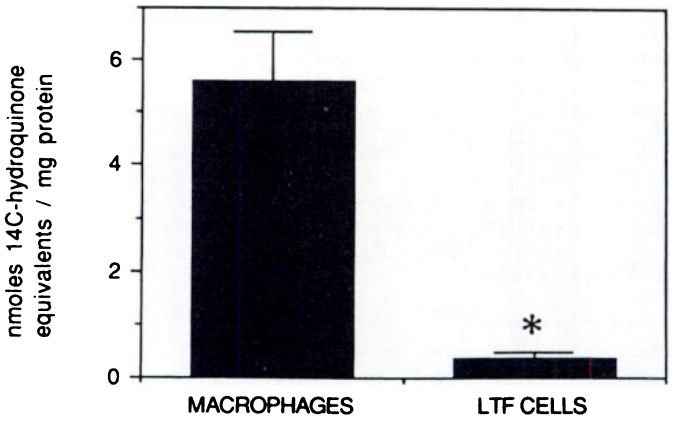

Fig. 1. Covalent binding of [^{14}C]hydroquinone to cellular macromolecules in macrophage cultures versus LTF cultures. Day 5 macrophages and confluent cultures of LTF cells were prepared and incubated with radiolabeled hydroquinone for 24 hr at 37° in 5% CO_2/95% O_2. Reactions were terminated by the addition of PCA. The amount of radiolabel covalently bound to cellular macromolecules was determined. Data are expressed as nanomol of [^{14}C]hydroquinone equivalents/mg of protein ± standard error for each cell type for five samples. *Significantly different from macrophage cultures ($p \leq 0.05$), as determined by *t* test analysis.

The ability of cellular homogenates to bioactivate radiolabeled hydroquinone in the presence of increasing concentrations of hydrogen peroxide was also evaluated (Fig. 2). The results from this experiment indicate that, when increasing concentrations of peroxide were added, a peroxide concentration-dependent bioactivation of hydroquinone was observed in macrophage homogenates but not in LTF cell homogenates. In addition, maximal bioactivation in macrophage homogenates was 4-fold higher than in LTF cell homogenates when 40 μM hydrogen peroxide was used. No difference in bioactivation of hydroquinone was noted between the cell types in the absence of hydrogen peroxide. Autoclaved homogenates of macrophages and LTF cells assayed in the presence of peroxide were used as controls to monitor hydroquinone autooxidation. Autooxidation in both macrophages and LTF cells resulted in binding levels less than 1 nmol of [^{14}C]hydroquinone equivalents/mg of protein.

Macrophages contained detectable peroxidase activity, whereas LTF cells did not (Table 1). Sodium azide treatment, which inhibits peroxidase activity, eliminated the peroxidase activity in the macrophage homogenates. To support the role of peroxidase in the bioactivation of hydroquinone, experiments utilizing human MPO were performed. The results revealed a time-dependent increase in the amount of covalently bound radiolabel in the samples containing hydrogen peroxide and MPO that was not noted in samples without hydrogen peroxide and/or MPO (Fig. 3). At 30 min of incubation, binding was 4-fold higher in the MPO plus peroxide treatment group, compared with the other group. In addition, increased levels of covalent binding of ^{14}C were noted when higher concentrations of MPO were used (data not shown). The binding species was confirmed as 1,4-benzoquinone in experiments utilizing hydroquinone (0.1 mM), human MPO (0.25 units/ml), and hydrogen peroxide (0.12 mM) (Fig. 4). After 15 min of incubation, a mean of 97 nmol/ml (two experiments) hydroquinone was removed and a stoichiometric amount of 1,4-benzoquinone was formed. In control experiments in the absence of enzyme or in the absence of peroxide, greater than 91% of hydroquinone was still present after 15 min of incubation.

To confirm the identity of the binding species in macrophages, GSH was used as a nucleophilic trap. During incubation of macrophage homogenates with hydrogen peroxide (0.1 mM), hydroquinone (0.01 mM), and GSH (0.2 mM), a product corresponding to HQ-SG was detected using HPLC with electrochemical detection (t_r = 19.2 min) (Fig. 5). This product has previously been characterized electrochemically and chemically (39). The production of HQ-SG in macrophage incubations was dependent on the presence of macrophage homogenate and hydroquinone, and the product cochromatographed with the compound obtained from mixing 1,4-benzoquinone (0.01 mM) and [^{3}H]GSH (0.1 mM) in a cell-free system. Smaller amounts of conjugate were detected in the absence of added peroxide (25% of that obtained in the complete system) and in the absence of exogenous glutathione (38% of that obtained in the complete system), presumably arising from autooxidation of hydroquinone and endogenous GSH in macrophages, respectively. Similar results were obtained using a gradient HPLC

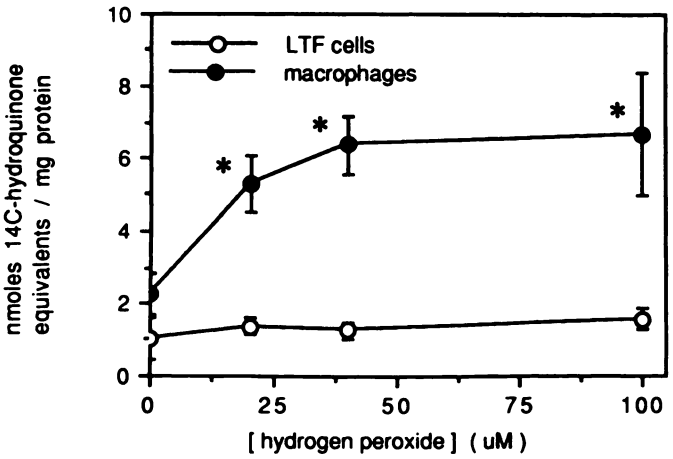

Fig. 2. Hydrogen peroxide-dependent binding of [^{14}C]hydroquinone to macromolecules in LTF cell or macrophage cell homogenates. Cell homogenates were prepared from cultures and were treated with radiolabeled hydroquinone for 30 min in the presence of increasing concentrations of hydrogen peroxide. Reactions were terminated by the addition of PCA. The amount of radiolabel covalently bound to cellular macromolecules was determined. Data are expressed as nanomol of [^{14}C] hydroquinone equivalents/mg of protein ± standard error for each cell type for four samples. *Significantly different from LTF cells treated with equal concentrations of hydrogen peroxide and significantly different from macrophages, [H_2O_2] = 0 ($p \leq 0.05$), as determined by two-way analysis of variance.

TABLE 1

Peroxidase activity in macrophages and LTF cells

Peroxidase activity was determined as the ability of cell homogenates to oxidize o-dianisidine in the presence of hydrogen peroxide. One unit of activity was defined as that degrading 1 μmol of peroxide/min at 25°. Data represent mean ± standard error for three experiments. Where shown, sodium azide was added to cell homogenates and incubated for 1 min before the addition of substrate solution.

Cell type	Peroxidase activity
	units/10^7 cells
Macrophages	0.014 ± 0.002
Macrophages + 100 μM azide	ND[a]
LTF cells	ND

[a] ND, none detected, with limits of detection equal to 0.002–0.0004 units/10^7 cells.

To confirm the identity of the binding species in macrophages, GSH was used as a nucleophilic trap. During incubation of macrophage homogenates with hydrogen peroxide (0.1 mM), hydroquinone (0.01 mM), and GSH (0.2 mM), a product corresponding to HQ-SG was detected using HPLC with electrochemical detection (t_r = 19.2 min) (Fig. 5). This product has previously been characterized electrochemically and chemically (39). The production of HQ-SG in macrophage incubations was dependent on the presence of macrophage homogenate and hydroquinone, and the product cochromatographed with the compound obtained from mixing 1,4-benzoquinone (0.01 mM) and [^{3}H]GSH (0.1 mM) in a cell-free system. Smaller amounts of conjugate were detected in the absence of added peroxide (25% of that obtained in the complete system) and in the absence of exogenous glutathione (38% of that obtained in the complete system), presumably arising from autooxidation of hydroquinone and endogenous GSH in macrophages, respectively. Similar results were obtained using a gradient HPLC

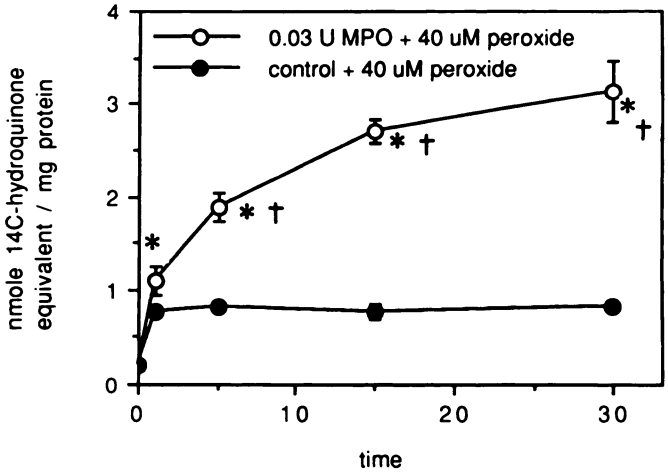

Fig. 3. Effect of time and peroxide on the MPO-mediated bioactivation of hydroquinone. Incubations consisted of 1.6–1.8 mg of ovalbumin, 10 μM radiolabeled hydroquinone, 0 or 0.03 units of MPO, and 0 or 40 μM hydrogen peroxide. The amount of covalently bound hydroquinone was determined after incubation for the indicated time. At time = 30 min, samples containing neither MPO or peroxide or samples containing only MPO had low levels of covalent binding (<0.75 nmol of [^{14}C]hydroquinone equivalents/mg of protein). Data are expressed as nanomol of [^{14}C] hydroquinone equivalents/mg of protein ± standard error for three or four samples. *Significantly different from time equals 0 for 0.03 units of MPO plus peroxide ($p \leq 0.05$), as determined by analysis of variance . †Significantly different from corresponding control plus peroxide ($p \leq 0.05$), as determined by two-way analysis of variance.

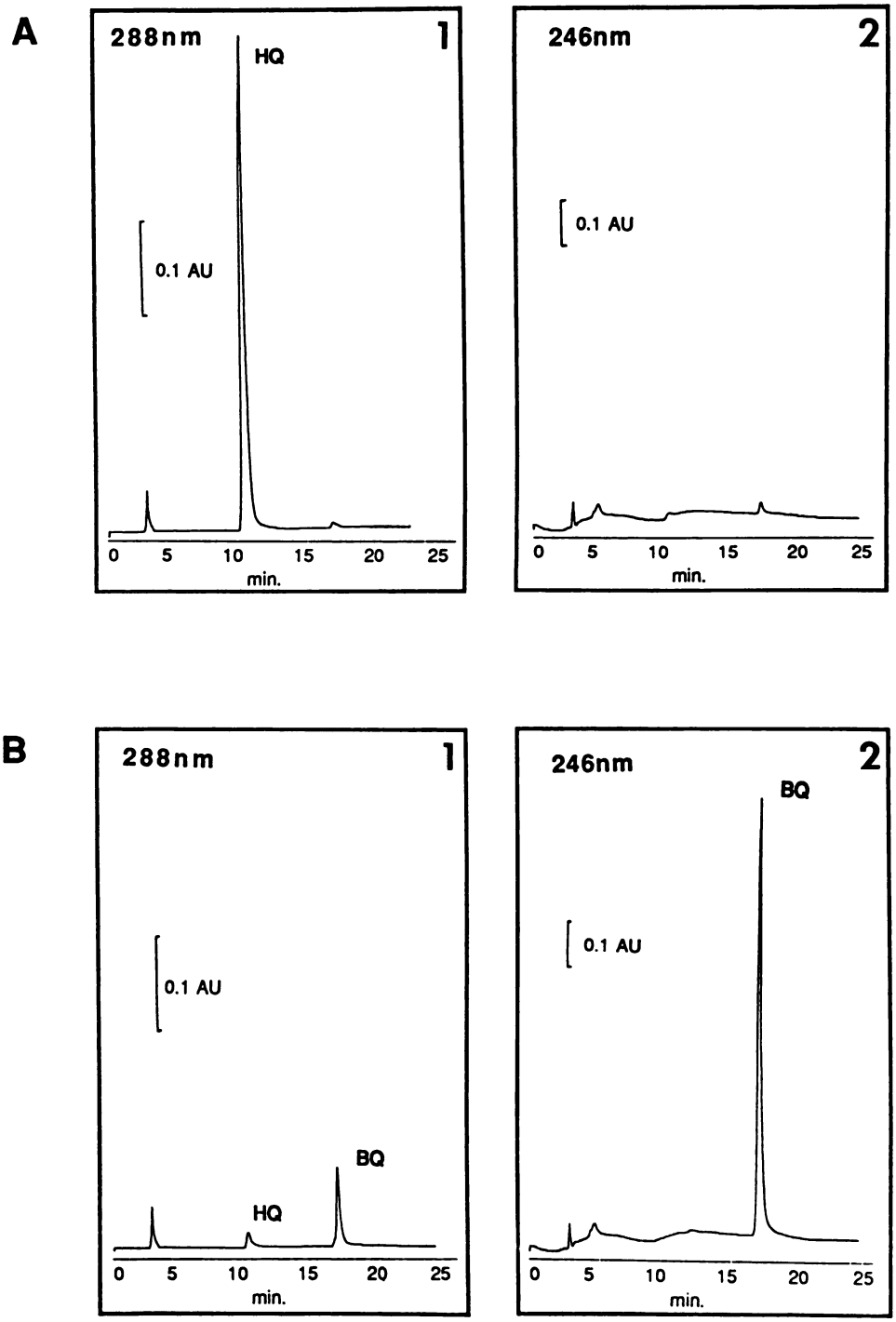

Fig. 4. Removal of hydroquinone (*HQ*) and formation of 1,4-benzoquinone (*BQ*) by MPO. HPLC analyses in the absence (A) and presence (B) of MPO were performed as described in Materials and Methods. Conditions: hydroquinone (0.1 mM), MPO (0.25 units/ml), and H_2O_2 (0.2 μM). Detection wavelengths of 288 and 246 nm were used to monitor hydroquinone disappearance and 1,4-benzoquinone appearance, respectively.

elution system coupled with UV/visible detection (see Materials and Methods). These data confirm that 1,4-benzoquinone is a reactive metabolite of hydroquinone produced in macrophages.

In order to investigate the possible role of deactivation processes in the cell-specific effects of hydroquinone, the activity of DT-diaphorase was examined in both LTF cells and macrophages. LTF cells contain approximately 4-fold higher DT-diaphorase activity than macrophages (Table 2).

Discussion

Although it is accepted that benzene requires metabolic activation in order to exert myelotoxicity, the metabolites responsible and their sites of generation remain unclear. Previous experiments in our laboratory indicated that treatment of bone marrow-derived stromal cells with the benzene metabolite hydroquinone reduces the ability of stromal cells to support myelopoiesis. Subsequent experiments revealed that the sensitivity of macrophages to the toxic effects of hydroquinone was much greater than that of LTF fibroblastoid cells (13). The toxicity to macrophages may be a direct effect of hydroquinone or may result from compounds formed by macrophage-mediated metabolism. Post *et al.* (42) and Schlosser and Kalf (43) demonstrated that peritoneal macrophage lysates had the ability to convert hydroquinone to a compound capable of covalently binding cellular macromolecules. Macrophages have been

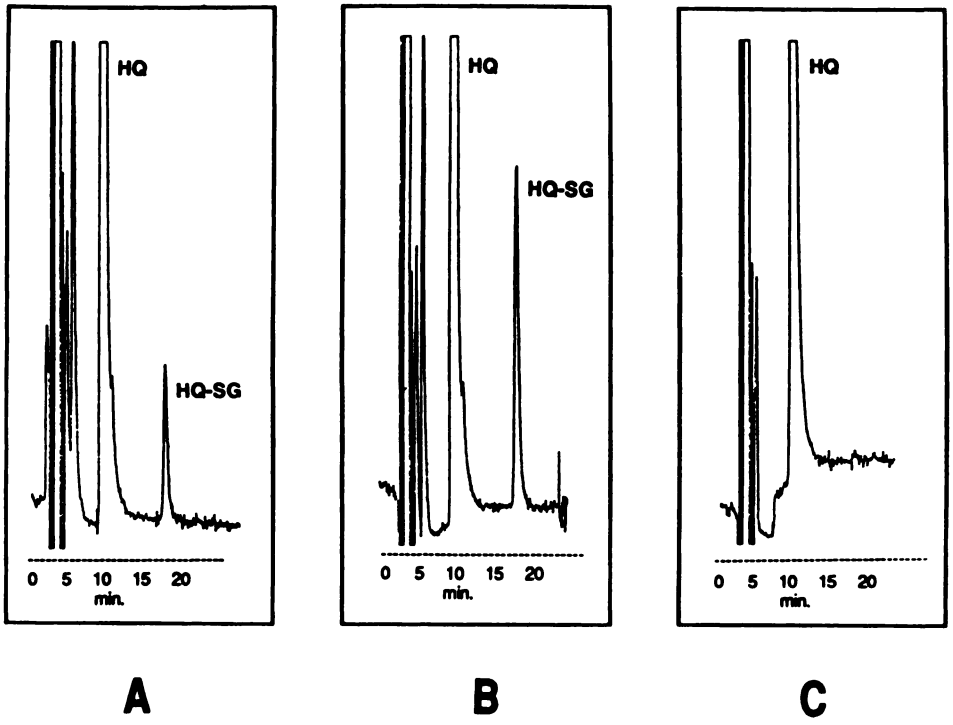

Fig. 5. Formation of a glutathione conjugate of hydroquinone (HQ-SG) in macrophage homogenates. Conditions: hydroquinone (*HQ*) (0.01 mM), GSH (0.2 mM), macrophage homogenate (equivalent to 0.18 mg of protein/ml), and H_2O_2 (0.1 mM). A, Complete system; B, as in A but plus aliquot of a reaction mixture of 1,4-benzoquinone (0.1 μM) and GSH (1 mM); C, as in A but minus the macrophage homogenate. HPLC analysis were performed as described in Materials and Methods (full scale deflection on the chromatograms shown = 40 nA).

TABLE 2

DT-diaphorase activity in macrophages and LTF cells

DT-diaphorase activity was determined by dicumarol-inhibitable reduction of dichlorophenolindophenol, as described in Materials and Methods. Results represent mean ± standard error of five separate experiments.

Cell type	DT-diaphorase activity
	nmol/min/mg of protein
Macrophages	25.6 ± 1.7
LTF cells	103.6 ± 4.0*

*$p < 0.05$ versus macrophages.

shown to contain peroxidase enzymes (17, 42–44) and such enzymes have the ability to convert polyhydroxy benzene metabolites into reactive molecules.

To address whether a similar mechanism is involved in the macrophage-selective effects of hydroquinone, cultures of bone marrow-derived macrophages and LTF cells were exposed to radioactive hydroquinone. The ability of each cell population to bioactivate hydroquinone was determined based upon the extent of covalent binding of radioactivity to cellular macromolecules. After 24 hr in culture, macrophages had significantly higher levels of radioactivity covalently bound to macromolecules than did LTF cells. The importance of peroxidases in the bioactivation process was supported by the observation that hydrogen peroxide-dependent covalent binding of radioactive hydroquinone occurred in homogenates of macrophages but not in homogenates of LTF cells. These results were consistent with the observation that homogenates of macrophages contained detectable peroxidase activity whereas LTF cells did not. The data are also consistent with the hypothesis that, in whole cells, macrophage-produced peroxide or hydroquinone autooxidation products acted as a peroxidase substrate, promoting the enzymatic conversion of hydroquinone to covalently binding species.

These results suggested that macrophages were selectively sensitive to the effects of hydroquinone because of their ability to bioactivate hydroquinone using peroxidase enzymes. Although the identity of the peroxidase(s) in macrophages is unclear, MPO represents one of the major peroxidases contained in whole bone marrow (45). Covalent binding experiments using purified MPO further support the role of a peroxidative metabolism in the bioactivation of hydroquinone. Oxidation of hydroquinone by MPO results in the formation of 1,4-benzosemiquinone, which disproportionates to 1,4-benzoquinone and hydroquinone (46).

When hydroquinone was incubated with MPO and hydrogen peroxide, the hydroquinone removed could be detected in stoichiometric amounts as 1,4-benzoquinone. When similar experiments were performed using macrophages, no 1,4-benzoquinone was detected.[3] This difference can be explained by the fact that the macrophage homogenates contained large quantities of macromolecules for binding of the reactive metabolite. In contrast, only microgram quantities of protein were available to covalently bind 1,4-benzoquinone when purified MPO was used. This would permit detection of this reactive species in incubations containing MPO but not homogenates. To confirm the identity of 1,4-benzoquinone as a reactive species formed during oxidation of hydroquinone in macrophage incubations, reactions were performed in the presence of excess GSH. This procedure traps the reactive quinone as its GSH conjugate. The HPLC method used was that of Lunte and Kissinger (39), who characterized HQ-SG by both electrochemical and chemical methods. That the product obtained using our conditions was indeed HQ-SG was confirmed by its similar chromatographic properties to those described by Lunte and Kissinger (39) and the observation that the putative conjugate cochromatographed with the product of the chemical reaction of 1,4-benzoquinone and GSH. These data confirm that 1,4-benzoquinone is a

[3] Unpublished observations.

reactive metabolite derived from hydroquinone in bone marrow macrophages.

Benzoquinone is capable of altering a broad range of macrophage functions including hydrogen peroxide release, interferon-priming, lipopolysaccharide-mediated activation of tumor cell cytolysis, and Fc receptor-mediated phagocytosis (47). Benzoquinone may also alter the ability of macrophages to regulate growth factor production. The toxic effects of benzoquinone may result from any of a number of mechanisms. Benzoquinone covalently binds to protein or to glutathione. For example, when administered to animals, benzoquinone reacts rapidly with blood and plasma proteins (48). In addition, 1,4-benzoquinone may bind covalently to nucleic acids (49). Covalent binding to DNA, RNA, or protein may interfere with DNA replication, RNA transcription and translation, and/or protein processing.

Quinones, in general, are known to undergo redox cycling reactions, which result in the generation of superoxide radical and other reactive oxygen species that are potentially toxic. Redox cycling reactions are unlikely to play a significant role in the toxicity of 1,4-benzoquinone, however, because the reaction of 1,4-benzoquinone with superoxide anion radical to form 1,4-benzosemiquinone occurs more readily than the reverse reaction (46).

The enzyme DT-diaphorase, a quinone reductase that plays a major role in quinone detoxification, is responsible for catalyzing a two-electron reduction of quinones to generate their hydroquinone derivative, which can be more readily excreted (40, 50, 51). As well as removing a potentially reactive quinone from a biological system, two-electron reduction bypasses semiquinone radical formation and the subsequent generation of reactive oxygen species. DT-diaphorase is, therefore, considered as a protective enzyme against quinone-induced toxicity. In our studies we found that LTF cells had 4-fold greater DT-diaphorase activity than macrophages. Both higher peroxidase activity and lower DT-diaphorase activity may, therefore, be involved in the selective sensitivity of macrophages to hydroquinone. This is in agreement with the results of Smart and Zannoni (52), who showed that the levels of both peroxidase and DT-diaphorase could influence the covalent binding of metabolites of [^{14}C]phenol in hepatic microsomes isolated from guinea pigs. Twerdok and Trush (53) found more DT-diaphorase activity in stromal cells of C57BL/6 mice, which are resistant to benzene, than the more sensitive DBA strain. These data, together with the results presented in our study, suggest that DT-diaphorase levels may be a critical determinant of the sensitivity of bone marrow cell types to the phenolic metabolites of benzene.

In summary, these studies, together with data from previous studies (13, 54), indicate that macrophages may be selective targets for benzene toxicity within the bone marrow stroma. This selectivity is postulated to be due, in part, to the selective ability of macrophages to bioactivate hydroquinone and/or the selective inability to detoxify 1,4-benzoquinone.

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