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Hydroquinone Inhibits Bone Marrow Pre-B Cell Maturation *In Vitro*

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SUMMARY

Environmental exposure to benzene results in both myelotoxicity and immunotoxicity. Although benzene-induced immunotoxicity has been well documented, no studies to date have addressed the possibility that benzene toxicity is due in part to altered differentiation of marrow lymphoid cells. We investigated the effect of acute exposure to the benzene metabolite, hydroquinone, on murine bone marrow B-lymphopoiesis. Bone marrow cell suspensions from B6C3F1 (C57BL/6J × C3H/HeJ) mice were depleted of mature surface IgM$^+$ (sIgM$^+$) B cells and cultured for 0, 24, 48, or 72 hr and production of newly formed B cells was assayed both by sIgM expression and colony formation in soft agar cultures. One hr exposure of bone marrow cells to hydroquinone before culture reduced the number of sIgM$^+$ cells generated in liquid cultures. Small pre-B cells (cytoplasmic μ heavy chain$^+$, sIgM$^-$) were numerically elevated as compared with control cultures. Hydroquinone exposure also decreased the number of adherent cells found in cultures of bone marrow cells. These results suggest that short-term exposure to hydroquinone, an oxidative metabolite of benzene, may in some way block the final maturation stages of B cell differentiation. This apparent differentiation block resulted in reduced numbers of B cells generated in culture and a corresponding accumulation of pre-B cells. Reduction of adherent cells in treated cultures may also suggest that toxicity to regulatory cells for the B lineage may be in part responsible for this aspect of hydroquinone myelotoxicity.

Benzene is a widely used industrial and commercial solvent. Exposure to benzene is known to result in a variety of blood dyscrasias including aplastic anemia, lymphocytopenia, and acute myelogenous leukemia (1). Although all hemopoietic lineages are thought to be susceptible to benzene toxicity, lymphoid cells appear to be particularly sensitive (2–4). Benzene myelotoxicity and immunotoxicity are apparently due to the oxidation metabolism of benzene to phenol, hydroquinone, catechol, benzoquinone, benzenetriol, and *trans*-muconaldehyde (1, 5–8). At least two of these metabolites, hydroquinone and catechol, have been shown to concentrate in the bone marrow of exposed animals (9, 10). This local concentration of benzene metabolites may result in targeted exposure of hemopoietic precursor cells to these toxic compounds. Hydroquinone and catechol have demonstrated toxicity to bone marrow myeloid cell growth *in vitro* (11). Administration of phenol, hydroquinone, or catechol to mice also significantly inhibits erythropoiesis (12), and acute doses of hydroquinone or catechol reduce the frequency of mitogen-responsive B-lymphocytes in both marrow and spleen (13).

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The reasons for this hematotoxicity of benzene, particularly the sensitivity of lymphocytes to benzene exposure, are unknown but probably involve inhibition of the development of hemopoietic cells (13–15). B-lymphocytes develop exclusively in the marrow and offer a particularly important model for studies of the relationship of benzene myelotoxicity to its immunotoxicity. B-lymphocytes are produced continually in large numbers in adult bone marrow (16), and intermediate stages of B lineage development can be identified by sequential changes in cell size and immunoglobulin expression (17). Large pre-B cells which express cytoplasmic heavy chains of IgM (cμ^+) are rapidly proliferating cells and divide to produce small pre-B cells. Small pre-B cells mature without cell division to express sIgM$^+$. This differentiation and maturation of B-lymphocyte precursors is thought to depend on regulatory cells present in the bone marrow hemopoietic microenvironment (18–21).

We questioned whether the observed immunotoxicity of benzene might be related to its effect on the development of early B-lymphocyte precursors. Experiments described in this report demonstrate that short-term, *in vitro* exposure (1 hr) of bone marrow cells to hydroquinone appear to block the maturation

ABBREVIATIONS: cμ^+, cytoplasmic heavy chain of IgM; sIgM$^+$, surface immunoglobulin M$^+$; FBS-HI, heat-inactivated fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; BL-CFC, B-lymphocyte colony formation in culture; LPS, lipopolysaccharide.

of B-lymphocytes from pre-B cells in culture. Hydroquinone exposure also decreased the number of adherent cell clusters found in cultures, and this reduction correlated well with the observed inhibition of B cell formation.

Materials and Methods

Animals. Male B6C3F1 (C57BL/6J female × C3H/HeJ male) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice used for experiments were 10–12 weeks old. All mice were housed in the West Virginia University Medical Center animal facility in microisolator cages (Lab Products, Rockville, MD). A 12-hr light cycle was maintained with an average temperature of 21 ± 5°. Animals had free access to food and water at all times.

Cell suspensions. Marrow cells were obtained by flushing femurs and tibias with ice-cold RPMI 1640 (Whittaker Biological, Walkersville, MD) containing 10% FBS-HI, and 25 mM HEPES buffer. Large debris was removed from the cell suspension by sedimentation over fetal bovine serum. Cells were transferred to a second tube containing a fetal bovine serum underlayer and centrifuged at 400 × *g* for 7 min at 4°. Cell viability was measured by trypan blue exclusion.

Chemicals. Hydroquinone or catechol (Sigma Chemical Co., St. Louis, MO) was diluted in sterile saline. Cells were diluted to 10^6/ml in PBS/5% FBS-HI, pulsed with drug for 1 hr, centrifuged, and resuspended in fresh medium.

Cell depletion. $sIgM^+$ cells were depleted from bone marrow cell suspensions by incubation on antibody-coated polystyrene plates exactly as described previously (22). Briefly, plates were incubated with 50 μg of affinity purified rabbit anti-mouse IgM (Pel-Freeze Biologicals, Rogers, AR) in 0.5 M Tris buffer, pH 9.5, at room temperature. Antibody-coated plates were incubated with PBS containing 5% fetal calf serum to block nonspecific binding sites on the plate. Cell suspensions (2×10^7/plate in 3 ml) were incubated on antibody-coated plates for 40 min at 4°, swirled, and incubated for an additional 30 min. Nonadherent cells were gently washed from the plates with ice-cold PBS containing 1% FBS-HI.

Detection of functional B-lymphocytes. Following 0, 24, 48, or 72 hr of incubation, functional B cells were assayed by their ability to proliferate and form BL-CFC (22–24). B cells were cloned in 0.4% Bacto-agar in supplemented McCoy's modified 5a medium containing 25 μg/ml LPS (*S. typhosa*, Sigma Chemical Co.). LPS is a mitogen that activates mouse B cells that have expressed surface immunoglobulin. Colonies were counted after 6 days in culture.

Immunofluorescent staining of B-lymphocytes. Bone marrow cells from liquid culture were stained for sIgM by incubation with fluorescein isocyanate-conjugated goat anti-mouse IgM (Southern Biotechnology Associates Inc., Birmingham, AL) for 20 min, washed with RPMI 1640 containing 0.08% sodium azide, and cytocentrifuged onto glass slides. Slides were blown dry and then fixed in 95% ethanol/5% glacial acetic acid for 10 min at −20° (25). To stain for cytoplasmic IgM, fixed cells were rehydrated in PBS, then incubated with rhodamine isocyanate-conjugated goat anti-mouse IgM (Southern Biotechnology Associates Inc.) in a humidified chamber for 20 min (25). Slides were washed extensively in PBS and mounted in elvanol (Fluoromount-G, Southern Biotechnology Associates Inc.). Fluorescent cells were

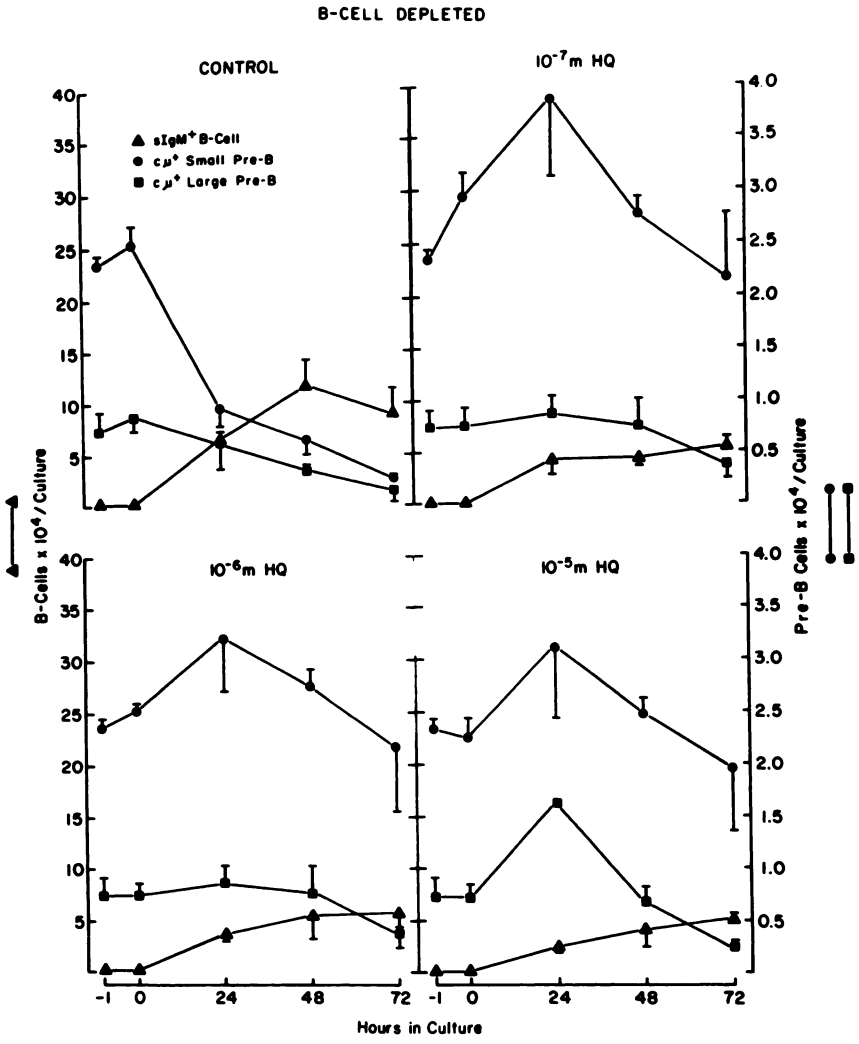

Fig. 1. Effect of 1 hr exposure to hydroquinone *in vitro* on pre-B cell ($c\mu^+$, sIg^-) and B cell ($sIgM^+$) number in B cell-depleted bone marrow cultures. Cell suspensions were removed from liquid culture at the indicated times and stained for Ig expression as described under Materials and Methods. Each point represents the mean value ± standard error of three separate experiments.

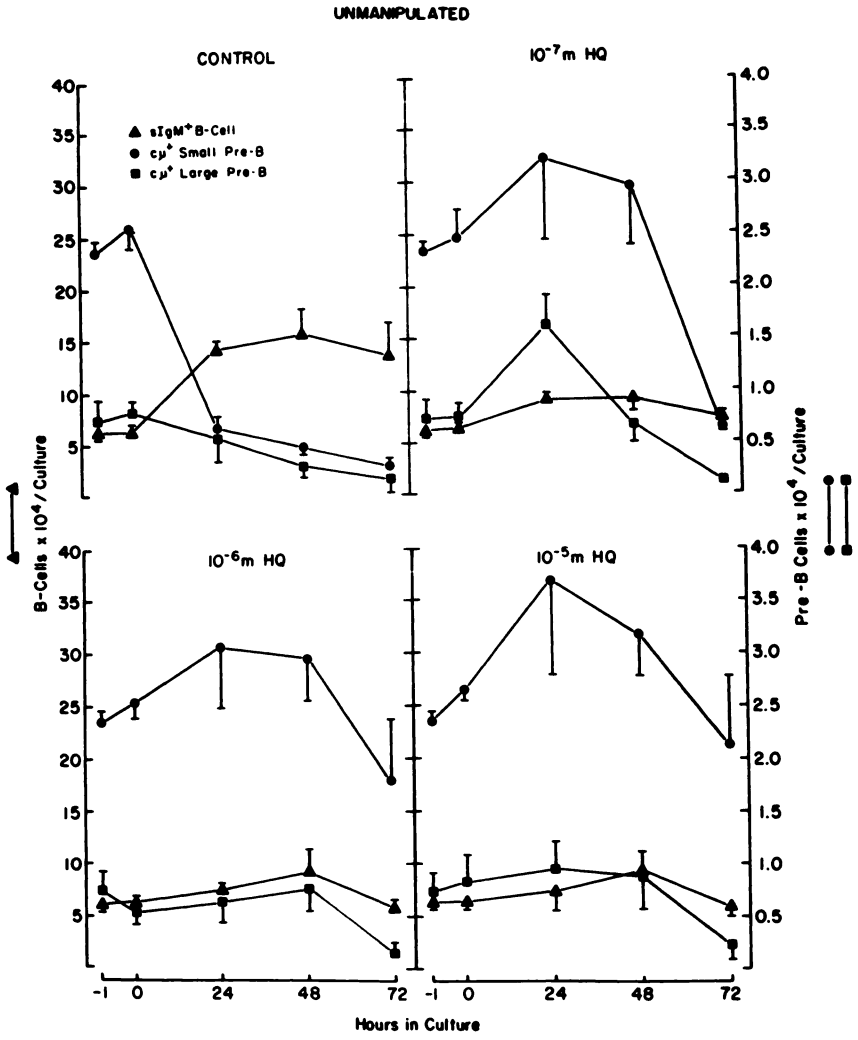

Fig. 2. Effect of 1 hr exposure to hydroquinone *in vitro* on pre-B and B cell number in unmanipulated bone marrow cultures. The procedure is identical to the one described in Fig. 1.

enumerated and sized using a Zeiss Universal microscope with epifluorescence.

Bone marrow-adherent cell staining. After removal of nonadherent cells and medium as previously described, adherent cell colonies were fixed and stained with Wright-Giemsa. Adherent cell clusters of >25 cells were counted using an inverted microscope.

Results

Phenotypic analysis of B-lymphocytes. Over the first 48 hr, sIgM$^+$ cells were generated in control cultures (Fig. 1). There was a corresponding decrease in small pre-B cells (cμ^+, sIgM$^-$, <9 μm diameter) in these cultures over the same period. Large pre-B cells (cμ^+, sIgM$^-$, >9 μm diameter) steadily declined over the period of culture. Treatment with hydroquinone (10^{-7}–10^{-5} M) for 1 hr delayed the observed decreases in small pre-B cell number in culture for at least 48 hr. This treatment also resulted in substantial reduction of newly generated sIgM$^+$ cells. sIgM$^+$ cells at 24 hr were 65%, 55%, and 40% of control values, whereas small pre-B cells were 403%, 333%, and 323% of control values after exposure to 10^{-7}, 10^{-6}, and 10^{-5} M hydroquinone, respectively. The number of large pre-B cells was essentially unaffected by hydroquinone exposure. A virtually identical pattern of altered B-lymphopoiesis was observed in marrow cultures that were not initially depleted of sIgM$^+$ cells (unmanipulated cultures; Fig. 2).

Analysis of bone marrow-adherent cells after hydroquinone exposure. Marrow-adherent cell clusters (>25 cells) were enumerated in control and hydroquinone-treated cultures. Data presented in Fig. 3 are representative of three separate experiments. Adherent cell clusters at 48 hr were 50%, 57%, and 36% of control; at 72 hr, they were 54%, 54%, and 57% of control after exposure to 10^{-7}, 10^{-6}, and 10^{-5} M hydroquinone, respectively.

Hydroquinone effect on BL-CFC. Depletion of B-lymphocytes removes cells which will respond immediately to LPS in agar cultures. However, B-lymphocyte precursors present in marrow mature during a period of liquid preculture to LPS responsiveness (Fig. 4). In control, unmanipulated cultures, BL-CFC frequency increased over the first 48 hr in culture and then declined slightly over the final 24 hr. Exposure of unmanipulated marrow cell suspensions to hydroquinone resulted in a concentration-dependent decrease in BL-CFC after 24 and 48 hr in culture when compared with corresponding control cultures (Fig. 4). These results were similar to those observed in B cell-depleted cultures (Fig. 4). The numbers of BL-CFCs in B cell-depleted cultures exposed to hydroquinone were 85%, 89%, and 90% of control at 24 hr, and 40%, 36%, and 30% of control at 48 hr after exposure to 10^{-7}, 10^{-6}, and 10^{-5} M hydroquinone, respectively. Hydroquinone-treated BL-CFC frequency approximated that of control after 72 hr in culture.

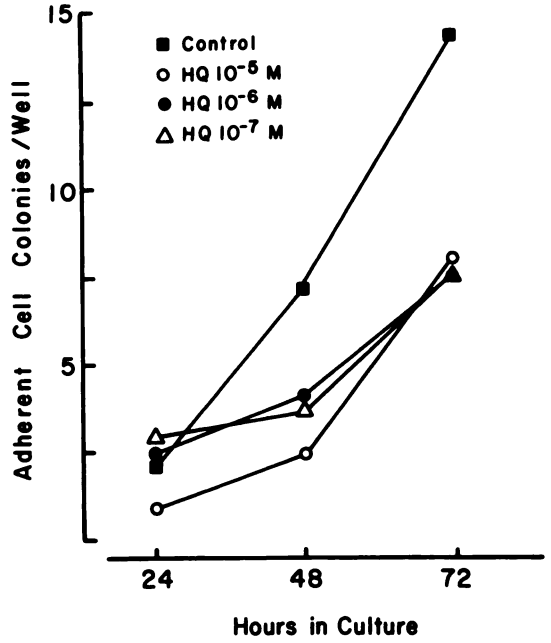

Fig. 3. Analysis of bone marrow-adherent cell cluster number. Bone marrow cell suspensions were exposed to the indicated concentrations of hydroquinone for 1 hr, washed, and recultured in fresh medium. Nonadherent cells were subsequently removed for analysis of B cell phenotype and function. Adherent cell clusters were then enumerated. Values represent mean cluster number in duplicate cultures ± standard error. Data are representative of three experiments.

Discussion

Benzene is both immunotoxic and carcinogenic in adult animals (4, 13, 26). Progressive B-lymphocytopenia following benzene exposure has been reported in studies of both rabbit blood (3) and mouse bone marrow (4). Reduction of lymphocyte populations may account for the impairment of normal immune function observed following chronic benzene exposure. Diminished lymphocyte numbers could result from either inhibition of cell proliferation or interference with bone marrow precursor cell development and maturation (3, 13) after exposure to either benzene (27) or its polyhydroxy metabolites (5, 26, 28).

In this study, hydroquinone reduced the frequency of functional B cells (BL-CFC) generated after 48 hr of culture by either unmanipulated or B cell-depleted bone marrow (Fig. 3). This period of culture has been previously shown to be optimal for pre-B cell maturation (18, 22). These data suggest that acute, low dose exposure to hydroquinone is toxic to B cell precursor development and that the affected population contains greater than half of the cells which can rapidly generate B cells *in vitro*. In addition, hydroquinone exposure did not affect LPS-stimulated B cell proliferation in unmanipulated cultures (Fig. 4). Previous studies demonstrated that concentrations of hydroquinone greater than 10^{-6} M are necessary to inhibit mitogen activation of lymphocytes (26, 29). Thus, the present studies rule out the possibility that immunotoxicity induced by 10^{-7} M hydroquinone is due to inhibition of mature B cell activation.

Phenotypic analysis of pre-B cell maturation in liquid bone marrow cultures suggested that observed hydroquinone effects on the generation of functional B cells (BL-CFC) stemmed from an inability of pre-B cells to mature into $sIgM^+$ B cells (Figs. 1 and 2). These data corroborate previous observations which suggested that *in vivo* benzene administration decreased the frequency of circulating lymphocytes and mitogen-induced blastogenesis of marrow B-lymphocytes (3, 4). In those studies 6-day exposure to 10 ppm benzene may have produced toxicity via inhibition of production of phenotypically and functionally identifiable B-lymphocytes.

Development of functional B cells from bone marrow precursors *in vitro* is dependent upon adherent accessory cells within the bone marrow (20). These adherent cells are collectively termed stromal cells, which are thought to form a hemopoietic inductive microenvironment which is important for the regulation of hemopoiesis (30). Previous studies have demonstrated that exposure of stromal cells to benzene metabolites results in a reduction in their ability to support granulocyte/monocyte colony formation in co-culture (31, 32). In the present study, hydroquinone reduced the number of adherent stromal cell colonies which develop in short-term liquid bone marrow cultures (Fig. 4). This decrease in adherent stromal cell colonies may account for the inhibition of pre-B cell maturation induced by hydroquinone. The precise mechanism involved is not yet known but may result from a reduction of pre-B cell differentiation factors normally produced by stromal cells or a reduction in direct cell-cell contact necessary for B cell generation (20, 21, 33).

It is important to note that the lowest concentration of hydroquinone used in these studies (10^{-7} M) effectively inhibited pre-B cell maturation. This is the same concentration of hydroquinone which is theoretically attained in humans exposed for 8 hr to 10 ppm (the current Occupational Health and Safety Administration threshold limit value) benzene in the work place (34).

In summary, we have shown that short-term exposure to hydroquinone, a polyhydroxy metabolite of benzene, inhibited marrow pre-B cell maturation. These observations provide evidence for the potential mechanism of immunotoxicity associated with benzene exposure and again emphasize that cells of the B lineage are particularly sensitive to benzene metabolites. It is not clear from these studies whether hydroquinone induces this effect by direct toxicity to the pre-B cell population or, in contrast, by toxicity to accessory cells necessary for pre-B cell maturation. Preliminary evidence suggests that hydroquinone-induced blockade of pre-B cell maturation may result from alterations in their interactions with adherent accessory cells. The precise nature of this adherent accessory cell alteration with respect to B-lymphopoiesis is currently under study in our laboratory. It should also be noted that these experiments involved acute exposure of marrow cells to hydroquinone. Chronic exposure to this compound, which more closely reproduces environmental exposure conditions, may further abrogate B cell generation. Benzene exposure is known to lead to leukemic transformation, and sustained blockade of differentiation in this lineage may significantly contribute to the development of carcinogenesis.

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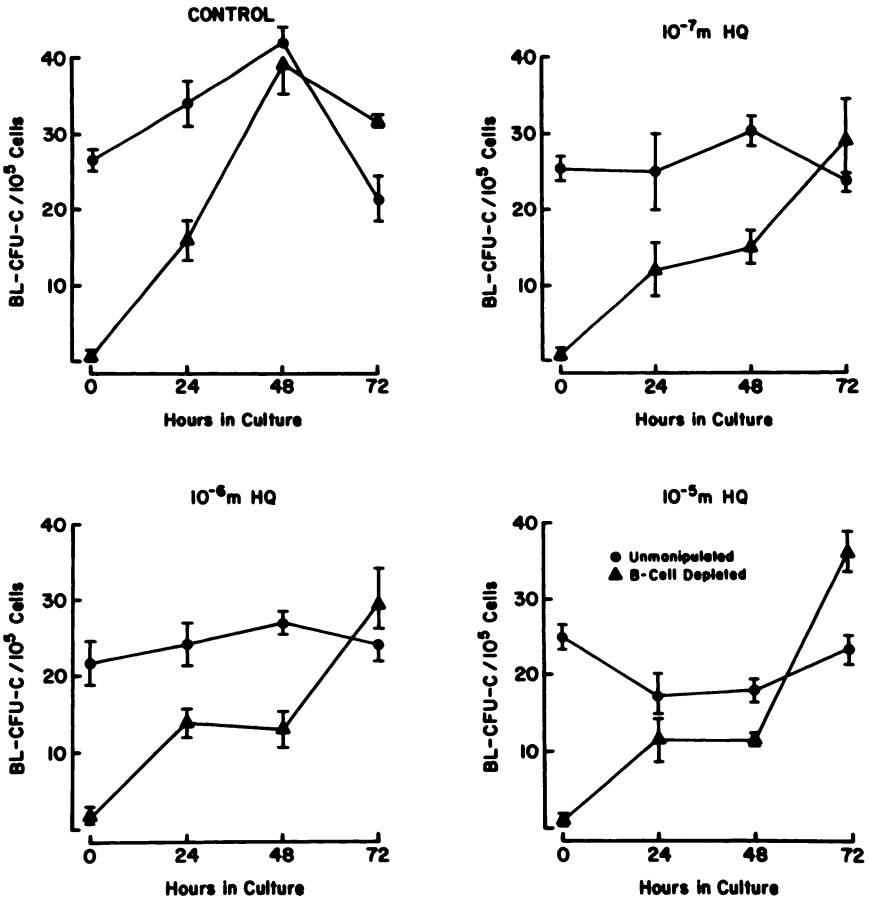

Fig. 4. Effect of 1 hr exposure to hydroquinone *in vitro* on BL-CFC in mouse bone marrow cultures. Cells from unmanipulated and B cell-depleted suspensions were removed from liquid culture at the indicated time intervals and further cultured in agar containing LPS for BL-CFC development. Each point represents the mean value of three separate experiments ± standard error.

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