Hematopoietic Effects of Benzene Inhalation Assessed by Long-term Bone Marrow Culture

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The strong and long-lasting hematotoxic effect after benzene exposure in vivo (300 ppm, 6 hr/day, 5 days/week for 2 weeks) was assessed in mice with bone marrow cells grown in long-term bone marrow culture (LTBMC). Bone marrow cultures initiated 1 day after the last benzene exposure did not produce adequate numbers of hematopoietic cells over 3 weeks and, in most cases, no erythroid or myeloid clonogenic cells could be recovered. The adherent cell layer of these cultures had a lower capacity for supporting in vitro hematopoiesis after the second seeding with normal bone marrow cells compared with control cultures. Two weeks after the last benzene exposure, body weight, hematocrit, bone marrow cellularity, and committed hematopoietic progenitor content (BFU-E and CFU-GM) were regenerated to normal or subnormal values, whereas hematopoiesis in LTBMC was very poor. Over 8 weeks, little or no significant committed progenitor production was observed. Treatment of mice exposed to benzene with heparin (three doses of 3 μg/g bw iv over 2 weeks for a total dose of 9 μg/g) partially overcame the toxic effect of benzene on the hematopoietic system as measured by the LTBMC method. Cultures from mice treated with heparin had a modest recovery of BFU-E and CFU-GM clonogenic potential after 5 to 6 weeks in LTBMC. In contrast, little or no recovery was obtained for the adherent cell layer clonogenic capacity, even after heparin treatment. These results clearly indicate a strong, long-lasting toxic effect on the bone marrow stroma and a limited recovery of hematopoietic potential by clonogenic cells of the nonadherent population after in vivo heparin treatment. — Environ Health Perspect 104(Suppl 6):1277–1282 (1996)

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Introduction

Benzene is a dangerous environmental toxin produced in nature and in large amounts by industry. Chronic exposure of animals to benzene is known to lead to progressive degeneration of the bone marrow and the drug metabolic system, leukopenia, aplastic anemia, and eventual leukemia (1–4). The clinical evidence indicating benzene as a primary inducer of diseases such as aplastic anemia and leukemia is well documented (5). Benzene can produce such toxic metabolites as phenol, catechol, and hydroquinone; apparently these agents can accumulate in the bone marrow (6–8). We have shown that hematotoxic targets for benzene and its metabolites may be related to depletion of heme and heme protein and induction of heme oxygenase (9). The direct effect of benzene on pluripotent stem cells (10,11) erythroid (12–14) and myeloid progenitors (15), lymphocytes (16), macrophages (17,18), and stromal elements (19) has been documented. Clonal cultures of bone marrow erythroid (BFU-E, CFU-E) and myeloid (CFU-GM) progenitors and spleen colony assays for pluripotent stem cells (CFU-S) have also verified the hematotoxic effect of benzene (11,12).

Although the mechanisms that induce hematotoxicity remain unclear, the apparent selectivity of benzene toxicity (or metabolites) for hematopoietic tissue may be connected with its capacity to be accumulated by bone marrow several times greater than that for other tissues (8). Depression of drug metabolism, growth factor production, and ribonucleic acid synthesis by cells of the hematopoietic microenvironment may also result from exposure to benzene or its metabolites (12,18,20). Furthermore, these metabolites can inhibit heme synthesis and induce the heme-degrading enzyme, heme oxygenase (12). Inadequate levels of heme may lead to a decrease in cytochrome P450 levels, disturbance of the drug-metabolizing system (12) and a variety of cellular disturbances throughout the hematopoietic system; these effects could be attributed to benzene. We have found that exogenous heme can counteract some toxic effects brought about by inhibition of heme synthesis by heavy metals and drugs (21–23), and more recently we have studied the effect of heme on hematopoiesis in long-term bone marrow culture (LTBMC) (24). Heme is an essential component of cytochrome P450 and the drug-metabolizing system and plays a central role in the regulatory network of heme synthesis under conditions of stress (25).

LTBMC represents a near-physiologic system for assessing growth characteristics of bone marrow cells in vitro. The proliferation and differentiation of stem cells are dependent on the intimate contact between the hematopoietic cells and a confluence of stroma (26). The importance of studying the long-term stromal effects of benzene is obvious, but effects on bone marrow growth in LTBMC have not yet been well characterized. Only a few reports have described benzene hematoxicity in LTBMC (27,28). These studies demonstrated a reduction in the CFU-S compartment in LTBMC derived from mice treated with benzene; this was thought to be due to the action of benzene on both hematopoietic and stromal cells. This conclusion was reached after coculture of normal hematopoietic cells on stromal-adherent cells derived from mice intoxicated with benzene and vice versa. Unfortunately, for the second seeding, a high dose of bone marrow cells (10^7) had to be used. It remains possible that a high inoculum of cells contributes to the regeneration of stromal progenitors for production of stromal adherent cells and overcomes the previous benzene toxicity.

Hemopoiesis probably has several beneficial effects on the hematopoietic system in normal and abnormal states. The aims of this project were to evaluate benzene toxicity to murine hematopoietic bone marrow stroma with LTBMC and to study the effectiveness of heme administration on hematopoietic regeneration in mice after benzene inhalation. A strong residual effect of benzene exposure on the capacity of hematopoietic cells to generate a favorable hematopoietic microenvironment in LTBMC was observed. Because heme has been shown to enhance hematopoietic progenitor proliferation and differentiation, as well as...
total cellularity, we examined the beneficial effect of hemin on clonogenic and stromal hematopoietic recovery. Results indicate partial recovery of erythroid and myeloid elements after hemin treatment, whereas damage to the adherent stromal cells was more permanent.

Methods
Male, specific pathogen-free DBA/2 mice, 10 to 14 weeks old (Charles River Laboratory, Charles River, Massachusetts), were used for benzene and control groups. For second seedings of bone marrow culture, female DBA/2 mice weighing 20 g and 8 to 12 weeks old were used. Mice used in each experiment were maintained under specific pathogen-free conditions.

Exposure to benzene was carried out in an isolation chamber system provided by C. Snyder of the New York University Department of Environmental Medicine. Chromatography-grade benzene was used for inhalation. Groups of 20 mice were exposed to conditioned air or to 300 ppm benzene for 6 hr/day, 5 days/week, 2 weeks.

Starting 2 days after the last benzene exposure, some of the mice intoxicated with benzene were given intravenous hemin injections (three doses of µg/g bw) over 2 weeks.

All bone marrow cultures were prepared under sterile conditions as described earlier (24,26,29). Methylcellulose techniques for erythroid (BFU-E) and myeloid (CFU-GM) colony assays have been described in detail (23). The nonadherent cells were counted and appropriate dilutions were made.

Results
Effect of in Vivo Benzene Exposure on Hematopoietic Indices
We examined the effect of benzene inhalation on hematopoietic compartments immediately after exposure or 12 days after the last exposure. As seen in Table 1, the body weights of DBA/2 mice exposed to 300 ppm benzene for 6 hr/day, 5 days/week for 2 weeks were decreased by 15% compared to body weights of the control group. Peripheral blood anemia and leukopenia were also observed, and hematocrits decreased to 74 to 76% of normal. Bone marrow cellularity was suppressed by as much as 93% in mice exposed to benzene. Furthermore, hematopoietic clonal efficiency was decreased and was seen as a reduction in the ability to generate BFU-E and CFU-GM. In particular, mice that inhaled benzene had diminished clonal capacity for BFU-E and CFU-GM content per tibia. This was most striking for BFU-E/tibia and was decreased by as much as 90% compared with control mice.

As shown in Table 1, significant regeneration of hematopoietic elements occurred after 2 weeks, and recovery of BFU-E to 96% of control levels was observed. In addition, bone marrow cellularity improved (68% of control) and CFU-GM (66% of control) regenerated after discontinuation of benzene exposure. Because hemin has been used to overcome bone marrow toxicity by exogenous chemicals and drugs (21-23), hemin was injected intravenously into mice during the recovery time at a dose of 3 µg/g bw, (over 2 weeks for a total dose of 9 µg/g bw), and hematopoietic indices were compared. This dose of hemin has been shown to be effective in myelodysplastic patients (30). Better recovery of all hematopoietic indices occurred in mice exposed to benzene and treated with hemin compared with mice subjected to benzene exposure alone (Table 1).

Effect of in Vivo Benzene Exposure on Stromal Adherent Cell Capacity to Support LTBM C
Control and benzene-exposed mice were killed 1 day after the last benzene exposure and bone marrow was used for LTBM C. Bone marrow cells from mice exposed to benzene were able to form an adherent cell layer in LTBM C, but production of the nonadherent cell population was almost negligible. Consequently, studies were conducted to evaluate the ability of the stromal adherent cells to support the proliferation and differentiation of normal hematopoietic cells. LTBM Cs from control mice and animals exposed to benzene were allowed to grow for 3 to 4 weeks, after which time the cultures were reseeded with normal bone marrow cells. The cultures were secondary-seeded with a single-cell suspension of 1.5 × 10⁶ normal bone marrow cells [the stromal cell content in the number of cells is not sufficient to provide an adequate hematopoietic microenvironment (31)]. Thus, cultures contained stromal adherent cells derived from cells of mice exposed to benzene and fresh cells from unexposed animals. The results are presented in Figure 1. In all cases we saw depression in cellularity and clonogenic potential in reseeded LTBM Cs grown in the presence of stromal adherent cells from mice that had previously been exposed to benzene.

Effect of in Vivo Benzene Exposure on Nonseeded LTBM C Capacity
LTBM Cs were set up 12 days after the last benzene exposure. Cumulative cell production during the next 4 to 8 weeks was suppressed in cultures obtained from bone marrow of benzene-exposed mice compared with controls (Figure 1). Figure 2A shows that the cellularity of control cultures for weeks 7 and 8 ranged between 12.5 and 16 × 10⁶ cells/culture for the same period.

Erythroid (BFU-E) and myeloid (CFU-GM) colony-forming progenitor cells were quantitated from LTBM Cs derived from controls and mice exposed to benzene. Control cultures exhibited significant cloning potential during weeks 4 through 8, whereas cultures derived from animals that inhaled benzene had negligible

Table 1. Effects of benzene exposure and hemin treatment on body weight and hematopoietic indices in mice (means ± SEM).

| Group         | Time after last exposure, days | Body weight, g | Hematocrit, % | Leukocytes | Bone marrow cellularity, ×10⁶ | BFU-E, per femur | CFU-GM, per femur |
|---------------|-------------------------------|----------------|---------------|------------|-------------------------------|-----------------|-------------------|
| **Experiment 1** |                               |                |               |            |                               |                 |                   |
| Control       | 1                             | 28 ± 1         | 44.1          | 3.0        | 11.0 ± 0.5                    | 3250 ± 34       | 13,640 ± 104      |
| Benzene       | 1                             | 24 ± 1         | 32.5          | 0.8        | 0.7 ± 0.3                     | 280 ± 15        | 7,140 ± 97        |
| **Experiment 2** |                               |                |               |            |                               |                 |                   |
| Control       | 12                            | 29 ± 1         | 44.2          | 4.7        | 11.3 ± 0.6                    | 791 ± 21        | 23,746 ± 111      |
| Benzene       | 12                            | 30 ± 1         | 39.1          | 1.9        | 7.7 ± 0.7                     | 760 ± 36        | 15,708 ± 87       |
| Benzene + hemin | 12                          | 30 ± 2         | 44.6          | 2.7        | 9.5 ± 1.0                    | 809 ± 42        | 23,370 ± 118      |

Indices were examined 1 and 12 days after benzene exposure (330 ppm, 6 hr/day, 5 days/week for 2 weeks). Hemin was given during recovery at 3 µg/g, iv, 3 times in 2 weeks.
Figure 1. Capacity of the adherent cell layer and the effect of reseeding on hematopoietic recovery in long-term bone marrow culture (LTBMC). LTBMCs of cells from controls and animals exposed to benzene (taken 1 day after last exposure) were allowed to grow for 3 to 4 weeks, after which time the cultures were reseeded with normal bone marrow cells. (A) Cumulative nonadherent cellularity; (B) cumulative erythroid progenitor (BFU-E) during weeks 5 to 7; (C) cumulative myeloid progenitor (CFU-GM) during weeks 5 to 7.

Figure 2. Effect of in vivo benzene exposure and hemin on long-term bone marrow culture (LTBMC) capacity. Cellularity and clonogenic potential were determined in LTBMCs with bone marrow from controls and animals exposed to benzene and then given hemin 12 days after the last benzene exposure. (A) Cumulative nonadherent cellularity during 8 weeks of culture; (B) cumulative erythroid progenitor production (BFU-E) during weeks 4 to 8 of culture; (C) cumulative myeloid progenitor production (CFU-GM) during weeks 4 to 8 of culture.

colony-forming potential (Figure 2B,C). During weeks 4 to 8, control BFU-E colonies/culture ranged from 1.3 to 6 × 10^2/culture and CFU-GMs ranged from 2.2 to 9 × 10^3/culture (Figure 2B,C). Almost no colony growth could be quantitated from cultures derived from animals exposed to benzene during the same culture period. Hence, the regeneration of stromal progenitors capable of producing functionally active stromal adherent cells was not complete 2 weeks after the last benzene exposure.

Effect of Hemin on Hematopoietic Recovery of Mice Intoxicated with Benzene

Cellularity and clonogenic potential were determined in LTBMCs with bone marrow from mice exposed to benzene and treated with hemin (Figure 2). In all cases, hemin was found to have significant beneficial effects on the capacity of LTBMCs to support bone marrow cell growth after mice had been exposed to benzene. During weeks 1 to 8 of culture, cultures derived from mice given hemin had increases in cellularity from 1.5 × 10^6 cells/culture (hemin) (Figure 2A). In addition, BFU-E and CFU-GM growth potential during weeks 4 to 8 increased from negligible values to 1.2 × 10^2 BFU-E/culture and 2.1 × 10^3 CFU-GM/culture (Figure 2B,C). Thus,
there was modest sparing of clonogenic potential in LTBMC by cells from animals exposed to benzene and treated with hemin.

Effect of Benzene and Hemin on Hematopoiesis by Adherent Cells

Hemin was given to mice treated with benzene 12 days after the last exposure to benzene in a manner similar to the procedure described in Table 1. Animals were then killed and LTBMCs were established for controls, mice exposed to benzene and mice exposed to benzene but treated with hemin. Cultures were grown for 8 weeks, after which time all of the adherent cells were removed from cultures, and cellularity and clonogenic potential were quantitated with only adherent cells. Results of these studies are depicted in Figure 3. The data demonstrate that some population of cells in the adherent layer of LTBMCs has the capacity to give rise to erythroid and myeloid clonogenic growth. Control cultures gave rise to approximately 7.0 ± 1 × 10^5 BFU-E/culture and 6.3 ± 1.1 × 10^3 CFU-GM/culture (Figure 3B,C). It is clear that cultures from animals exposed to benzene had marked reductions in the total number of adherent cells/culture. Note that the cellularity of control cultures was 15.6 ± 1 × 10^5 adherent cells/culture, whereas cellularity of cultures from mice exposed to benzene was 3.1 ± 1 × 10^5 adherent cells/culture (Figure 3A). In addition, Figure 3A shows that in vivo hemin treatment improved the cellularity of LTBMCs previously exposed to benzene. Results demonstrate that adherent cell numbers in cultures from animals exposed to benzene and treated with hemin were 6.9 ± 1.2 × 10^6 adherent cells/culture compared with 3.1 ± 1 × 10^6 adherent cells/culture for benzene exposure alone. No significant improvement was seen in BFU-E/CFU-GM clonogenic potential by adherent cells (slight improvement in BFU-GM clonogenic capacity was seen), but this did not appear to be significant (Figure 3B,C). Thus, in vivo hemin treatment was not as effective for hematopoietic recovery by adherent cells when contrasted with the nonadherent cell population shown in Figure 2.

Discussion

Excessive benzene exposure in humans and other mammals often results in disorders such as leukemia and aplastic anemia (3,5). Disturbed or impaired stromal cells may contribute to abnormal hematopoiesis and thus to the pathogenesis of these disorders (32,33). Results from our studies clearly demonstrate the long-term toxic effects of benzene on the hematopoietic stromal progenitors and the capacity to generate a viable adherent cell layer that can support sustained hematopoiesis. Hemin had some beneficial effects on the recovery of stromal capacity to generate an adherent cell layer able to support cellularity and clonogenicity by cells from LTBMC. However, adherent cell layers from mice exposed to benzene and treated with hemin recovered only partially both quantitatively and qualitatively; in these cultures, the relative content of clonogenic progenitors in stromal adherent cells with cells in suspension was sharply decreased. These toxic effects of benzene and the sparing influence of hemin are not obvious if one examines only the hematopoietic indices directly from mice after benzene exposure.

Inadequate production of specific growth factors or extracellular matrix substances by the stroma may be one of the damaging effects of benzene. It is not clear whether benzene itself is the primary toxin or whether more toxic metabolites such as phenol are responsible. We have demonstrated that phenol is more toxic to bone marrow CFU-E and stromal growth than benzene (12). Furthermore, benzene is known to promote a marked increase in bone marrow heme oxygenase activity, and this could contribute to our results (12). Hemin has been found to reverse some of the bone marrow toxicity caused by heavy metals (21) and drugs such as azidothymidine (AZT) (23). It is thought that the toxicity of these agents, in part, is caused by a disturbance in the heme biosynthetic pathway and a decrease in cellular hemoprotein, including the cytochrome P450 system. Thus, exogenous hemin can bypass the defective steps and restore adequate levels of heme (21) needed for cell function, including drug biotransformation.

Macrophages are a population of adherent cells with the capacity to metabolize benzene; cells of this type are included in the stromal adherent cell layer of LTBMCs (17,18). Macrophages and other adherent cells may also have the capacity to store or accumulate exogenous or endogenous metabolic products for extended periods of time (34). The lack of significant in vivo hemin response may, in part, be related to the formation of more toxic benzene metabolites in vivo. Damage to cells of the adherent cell population may thus be more permanent, as evidenced by a poor recovery in vitro. In this regard, benzene exposure in vivo produced cell populations that remained depressed in cellularity and clonogenic potential over the 8-week period of LTBMC. This is in marked contrast to results obtained with short-term CFU cultures performed 12 days after benzene exposure. As seen in Table 1, significant recovery of CFU potential/femur cellularity, hematocrit and body weight occurred; these results do not bear out the more permanent long-term effects of benzene. The more immediate recovery of CFUs as seen here may be derived mainly from cells of the nonadherent population in the bone marrow. Our results from LTBMCs support this inference (Figure 2).

A direct permanent cytotoxic effect of benzene on the hematopoietic stroma is
further evidenced by the reduced number of adherent hematopoietic cells obtained in LTBMCS from animals exposed to benzene. Previous studies suggest that some cells of the adherent hematopoietic cell layer of LTBMCS are actually a subpopulation of CFU-S with regard to pluripotency and proliferation capacity (35). Thus, a depression in adherent cell numbers by benzene or its metabolites would result in a permanent depression in stem cell numbers, limited capacity to regenerate progenitor cell compartments on demand, or both. This was seen as a marked depression of CFU potential by LTBMCS for up to 8 weeks in culture. Nevertheless, this extreme toxic effect does not express itself so severely in the more immediate population of nonadherent cells, which are probably in a more active phase of the cell cycle.

In conclusion, the strong hematopoietic residual effects of benzene were demonstrated in LTBMCS. Hematopoiesis in culture over 8 weeks was on the threshold level, and little or no progenitor production was observed by cells from animals exposed to benzene. Perhaps this concealed and long-lasting damage of hematopoietic and stromal progenitors is an important part of the leukemogenic activity of benzene. Furthermore, hemin may augment benzene detoxification and alleviate its cytotoxic effect on the hematopoietic microenvironment.

Irrespective of the mechanisms involved in the accelerated hematopoietic regeneration induced by hemin in mice exposed to benzene, the use of hemin along with or a combination of growth factors may be of clinical importance (25). In fact, hemin has a beneficial effect on improvement of the anemia seen in patients with myelodysplasia (30).

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