Role of Nitric Oxide in Hematosuppression and Benzene-induced Toxicity

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It is becoming increasingly apparent that nitric oxide plays a multifunctional role in regulating inflammatory processes in the body. Although nitric oxide and its oxidation products are cytotoxic toward certain pathogens, they can also cause tissue injury and suppress proliferation. Cytokines and growth factors released at sites of inflammation or injury stimulate both immune and nonimmune cells to produce nitric oxide. Nowhere in the body is this more detrimental than in the bone marrow, for the continuous production of hematopoietic precursors is essential for normal blood cell maturation. Our laboratories have discovered that, in response to inflammatory mediators, bone marrow cells readily produce nitric oxide. Nitric oxide production is enhanced by hematopoietic growth factors including interleukin-3, macrophage colony stimulating factor, and granulocyte–macrophage colony-stimulating factor. When bone marrow cells produce nitric oxide, hematopoiesis is impaired, an effect that is potentiated by colony-stimulating factors. Treatment of mice with benzene, which suppresses bone marrow cell development, was found to markedly enhance the ability of bone marrow cells to produce nitric oxide in response to inflammatory mediators alone and in combination with hematopoietic growth factors. Taken together, these data suggest that nitric oxide may be an important mediator of benzene-induced bone marrow suppression. — Environ Health Perspect 104(Suppl 6):1283–1287 (1996)

Key words: benzene, nitric oxide, bone marrow, GM–CSF, CSF-1, interleukin-3

Introduction

It is well recognized that growth factors and cytokines regulate hematopoiesis in the bone marrow. Granulocyte–macrophage colony-stimulating factor (GM–CSF), granulocyte CSF (G-CSF), macrophage CSF (CSF-1), and interleukin-3 (IL-3 or multi-CSF) are the most well characterized of the growth factors. By acting directly on progenitor cells, these cytokines have the capacity to induce bone marrow cell proliferation and maturation. In several experimental animal models, benzene or its metabolites induce a marked decrease in bone marrow cellularity as well as impaired host resistance (*1–6*). Although the mechanisms underlying the action of benzene are not known, a number of studies have suggested that stromal cells within the bone marrow are a sensitive target (*7*). Several reports have demonstrated that the function of stromal cells, which consist predominantly of mature macrophages, are reduced after exposure of mice to benzene or its metabolites. Thus, after benzene exposure, these cells are unable to support proliferation of stem cells or the development of hematopoietic progenitors (*8–11*). In addition, *in vitro* exposure of stromal cells to benzene metabolites such as hydroquinone, benzoquinone, catechol, and benzenetriol reduces their capacity to produce cytokines and regulate hematopoiesis (*11–15*). In contrast to these results, our laboratories have found that benzene treatment of mice leads to activation and/or increased maturation of bone marrow macrophages (*2,16,17*). These cells increase in number and produce hydrogen peroxide, interleukin-1, and tumor necrosis factor-α, each of which has been implicated in tissue injury and/or altered cellular proliferation (*18*). In the present studies, these observations were extended by examining the effects of benzene and its metabolites on nitric oxide production by bone marrow cells.

Nitric oxide is an important cytotoxic effector molecule synthesized by activated macrophages against intracellular pathogens (*19*). It is produced via the NADPH-dependent enzyme nitric oxide synthase by the oxidation of a guanidino nitrogen of L-arginine (*20*). Once formed, nitric oxide can complex with iron–sulfur or heme-containing proteins in cells, inhibiting and/or activating a variety of enzymes that can lead to reduced DNA synthesis and cytotoxicity (*19,20*). Nitric oxide also readily reacts with active oxygen, in particular, superoxide anion, resulting in highly toxic peroxynitrite radicals (*21*). We have found that benzene treatment of mice causes increased production of nitric oxide by bone marrow cells in response to inflammatory mediators and colony-stimulating factors. Moreover, nitric oxide production by bone marrow cells is inversely correlated with cellular proliferation. Cells from benzene-treated mice exhibited increased sensitivity to nitric oxide-mediated growth inhibition, a finding consistent with the idea that nitric oxide contributes to the reduced bone marrow cellularity and impaired hematopoiesis observed after benzene exposure (*7,22,23*).

Materials and Methods

Benzene and its metabolites were obtained from Fisher Scientific (Springfield, NJ). N^G-monomethyl-L-arginine (L-NMMA) was purchased from Chem-Biochem Research, Inc. (Salt Lake City, UT) and was greater than 99% pure. Murine rGM-CSF, rIL-3, tumor necrosis factor-α (TNF-α), and human rM-CSF and rIL-1β were from Genzyme Corp. (Boston, MA). Purified mouse recombinant interferon-γ (rIFN-γ) was generously provided by Sidney Pestka (UMDNJ-Robert Wood Johnson Medical School). Lipopolysaccharide ([LPS]; *Escherichia coli* serotype 0128:B12) and all other reagents were from Sigma Chemical Co. (St. Louis, MO). Reagents were diluted in medium and prepared fresh immediately before use.

Female Balb/c mice (20–25 g) were purchased from Taconic Farms (Germantown,

This paper was presented at Benzene '95: An International Conference on the Toxicity, Carcinogenesis, and Epidemiology of Benzene held 17–20 June 1995 in Piscataway, New Jersey. Manuscript received 16 January 1996; manuscript accepted 14 June 1996.

D.L. Laskin is a Burroughs Wellcome Toxicology Scholar. This work was supported by the Burroughs Wellcome Fund and by National Institutes of Health grants ES04738, ES03647, GM34310, and ES05022.

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Abbreviations used: CSF, colony-stimulating factor; G, granulocyte; M, macrophage; IL-3, interleukin-3; L-NMMA, N^G-monomethyl-L-arginine; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; LPS, lipopolysaccharide; HBSS, Hank's balanced salt solution; iNOS, inducible nitric oxide synthase.

NY). Animals were housed in microisolator cages and received food and sterile water *ad libitum.* Mice were injected ip with 800 mg/kg benzene (Fisher Scientific) in corn oil, 100 mg/kg hydroquinone, 25 mg/kg 1,2,4-benzenetriol, 2 mg/kg *p*-benzoquinone, or corn oil control once/day for 3 days or twice/day for 2 days. These treatment protocols were found to induce bone marrow suppression (*24–26*). In some experiments mice were also injected iv with L-NMMA (1.25–10 mg/mouse) before benzene treatment.

Eighteen hours after the last treatment with benzene, benzene metabolites, or control, mice were euthanized, the lower leg bones were removed, the femurs and tibias flushed with 5 ml of ice-cold Hank's balanced salt solution (HBSS), and the cell suspensions washed once in ice-cold HBSS. For determination of bone marrow cellularity, contaminating red blood cells were lysed using 0.75% ammonium chloride in 20 mM Tris-HCl buffer, pH 7.2. For all other experiments, the bone marrow cell suspension was layered over lymphocyte separation medium (Organon Teknika, Durham, NC) and centrifuged for 30 min at 85*g*, 20°C. This isolation procedure removed erythrocytes and approximately 50% of the granulocytes from bone marrow cell suspensions (*17*). The low-density fraction, which consisted of granulocytes (35–40%), mononuclear phagocytes (8–12%), lymphocytes (25–30%), and precursors (15–20%), as determined by antibody binding and flow cytometry (*17*), was then washed three times with cold HBSS and enumerated using a Coulter counter. Viability was assessed by Trypan Blue exclusion. Bone marrow cells from groups of four mice were pooled and analyzed as a group.

Bone marrow cells were inoculated into 96-well dishes ($0.5–1.0 \times 10^6$ cells/well) in 250 µl of phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Biocell Laboratories, Carson, CA), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml) with and without various concentrations of growth factors and/or cytokines. This culture medium contains 84 mg/ml of L-arginine. Nitric oxide, quantified by the accumulation of nitrite in the culture medium, was measured by a microplate assay based on the Griess reaction with sodium nitrite as the standard (*22,27*).

Bone marrow cells were inoculated into 96-well dishes (1×10^6 cells/well) in the presence or absence of various stimuli. After 19 hr of incubation, ^{3}H-thymidine (1 µCi/well, 2.0 Ci/mmol; NEN Research Products, Wilmington, DE) was added. Cells were harvested 5 hr later onto glass-fiber filter paper using a PHD cell harvester, and counted for radioactivity.

Data were analyzed using the Student's *t*-test. Results were considered statistically significant at $p < 0.05$.

Results

Effects of Inflammatory Mediators and Colony-stimulating Factors on Nitric Oxide Production by Bone Marrow Phagocytes

Both IFN-γ and bacterial-derived LPS readily stimulated nitric oxide production by bone marrow cells for periods up to 72 hr (Figure 1) (*22*). A marked synergism was observed with combinations of IFN-γ and LPS. In contrast, in the absence of stimulation, bone marrow phagocytes produced negligible amounts of nitric oxide. Nitric oxide production by bone marrow cells in response to LPS and IFN-γ depended on L-arginine in the culture medium and was inhibited by L-NMMA and L-canavanine, two L-arginine analogs that are potent inhibitors of nitric oxide synthase (*22*). The inhibitory effects of these analogs could be reversed by adding excess L-arginine to the cultures (*22*). In previous studies using Northern blot analysis and specific cDNA probes, we showed that IFN-γ and LPS increased expression of an inducible form (type II) of nitric oxide synthase in bone marrow cells (*3*). Taken together, these data demonstrate that nitric oxide produced by the bone marrow cells is formed from L-arginine via nitric oxide synthase.

We next analyzed the effects of the colony-stimulating factors IL-3, GM-CSF, and M-CSF on nitric oxide production by bone marrow cells (Figure 1) (*3,22*). Although none of these cytokines alone induced nitric oxide production by bone marrow cells, a marked synergism was observed when they were used in combination with LPS or IFN-γ (Figure 1) (*3,22*).

Effects of Inducers of Nitric Oxide on Proliferation of Bone Marrow Cells

In the bone marrow, GM-CSF, M-CSF, and LPS stimulated bone marrow cell proliferation, as measured by increases in cell number (*3,22*) and ^{3}H-thymidine incorporation (Figure 2). GM-CSF and M-CSF were more effective in stimulating proliferation than was LPS (Figure 2) (*3,22*). Treatment of the bone marrow cells with combinations of either LPS or IFN-γ and

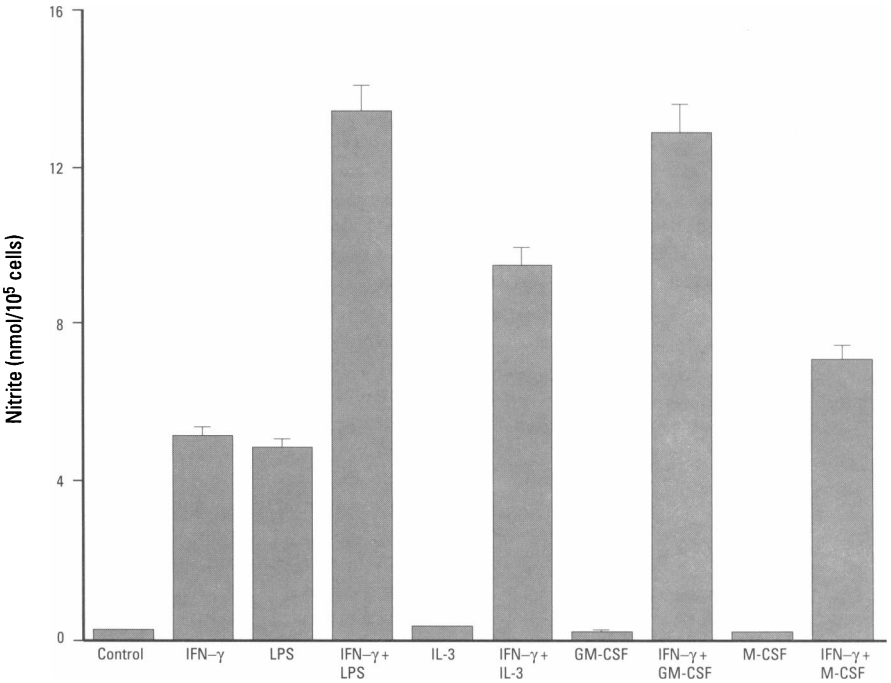

Figure 1. Effects of inflammatory mediators and colony-stimulating factors on nitric oxide production by bone marrow. Bone marrow cells were incubated with 100 U/ml IFN-γ, 1 µg/ml LPS, 100 U/ml IL-3, 200 U/ml GM–CSF, or 20 U/ml M-CSF, alone or in combination as indicated. After 72 hr, supernatants were collected and assayed for nitrite content. Each bar is the average ± SE of triplicate samples from one of three similar experiments.

GM-CSF or M-CSF, which stimulate nitric oxide production (Figure 1), resulted in a marked inhibition of bone marrow cell proliferation (Figure 2) (*3,22*). This inhibition was reversed by L-NMMA. A similar effect was observed in cells treated with combinations of CSF and IFN-γ (*3,22*).

Effects of Treatment of Mice with Benzene or Benzene Metabolites on Nitric Oxide Production by Bone Marrow Phagocytes

In our next series of studies, we analyzed the effects of benzene treatment of mice on bone marrow cell production of nitric oxide. As observed with cells from control animals, in the absence of stimulation, bone marrow cells from benzene-treated mice produced negligible quantities of nitric oxide. Treatment of these cells with LPS resulted in a dose and time-dependent increase in nitric oxide production (Figure 3) (*2,23*). Bone marrow cells from mice treated with benzene produced significantly more nitric oxide in response to LPS than did cells from control mice (Figure 3). We also found that cells from benzene-treated mice produced significantly more nitric oxide in response to combinations of LPS plus M-CSF or GM-CSF, as well as to IFN-γ alone and in combination with LPS than did cells from control animals (*3,23*). Taken together, these data indicate that benzene treatment of mice primes bone marrow cells to produce increased amounts of nitric oxide in response to inflammatory stimuli and growth factors. The fact that this was observed with both GM-CSF and M-CSF (*3,23*) suggests that the effects of benzene, with respect to nitric oxide production, are not cell-lineage specific.

In further studies, we found that increased production of nitric oxide was due to benzene-induced alterations in expression of inducible nitric oxide synthase in the bone marrow cells (*3*). As indicated above, no inducible nitric oxide synthase (iNOS) mRNA was detectable in unstimulated cells from either control or benzene-treated animals. However, treatment of the cells for 48 hr with the combination of LPS plus M-CSF induced expression of mRNA for iNOS. Scanning laser densitometry revealed that cells from benzene-treated animals produced 2-fold more iNOS mRNA than cells from control mice (*3*). These data are consistent with our results showing increased nitric oxide production by cells from benzene-treated mice (Figure 3). Thus, it appears that increased production of nitric oxide after benzene treatment of mice is due, at least in part, to enhanced expression of iNOS mRNA.

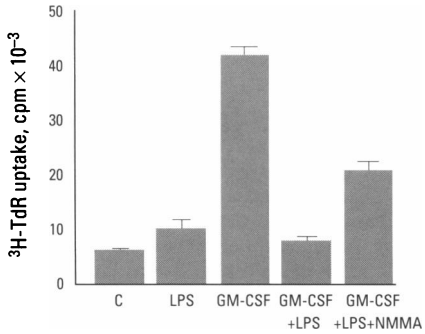

Figure 2. Effects of LPS and GM-CSF on bone marrow cell proliferation. Bone marrow cells were incubated with 1 μg/ml LPS or 200 U/ml GM-CSF alone or in combination with 1 mmol/liter L-NMMA as indicated. After 24 hr, tritiated thymidine uptake was measured as described in "Materials and Methods." Each bar is the average + SE of triplicate samples from one of three similar experiments.

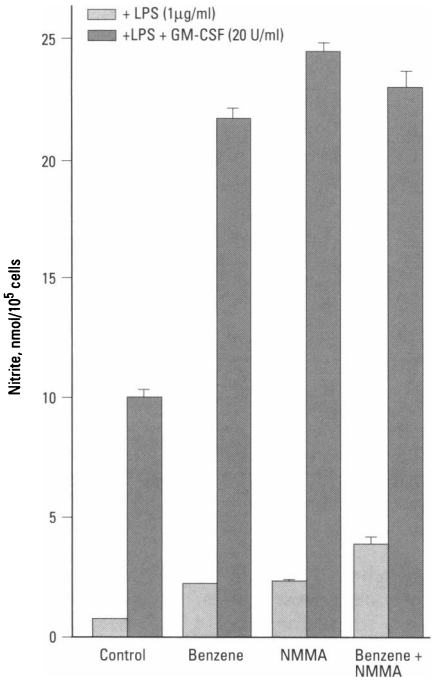

Figure 3. Effects of benzene and L-NMMA treatment of mice on nitric oxide production by bone marrow cells. Bone marrow cells from mice treated with corn oil (control), benzene, L-NMMA, or benzene + L-NMMA were incubated with LPS or LPS + GM-CSF as indicated. Supernatants were collected 72 hr later and analyzed for nitrite content. Each value is the average + SE of triplicate samples from one of three similar experiments. Cells treated with benzene both with and without L-NMMA were significantly ($p<0.05$) different from control mice.

We next examined the effects of treating mice with various metabolites of benzene on bone marrow cell nitric oxide production. As observed with benzene, treatment of mice with hydroquinone, *p*-benzoquinone, or 1,2,4-benzenetriol resulted in a marked increase in the ability of bone marrow phagocytes to produce nitric oxide in response to LPS and/or IFN-γ (*23*). Cells from hydroquinone or 1,2,4-benzenetriol treated mice produced more nitric oxide than cells from benzene or *p*-benzoquinone-treated mice. In addition, cells from mice treated with various benzene metabolites were more responsive to combinations of LPS and GM-CSF or M-CSF (*23*).

Effects of L-NMMA on Nitric Oxide Production by Bone Marrow Cells

As described above, nitric oxide production by bone marrow cells is inversely correlated with cellular proliferation. Our observation that cells from mice treated with benzene produce increased levels of nitric oxide in response to various stimuli suggested the possibility that L-NMMA might prevent benzene-induced decreases in bone marrow cellularity in mice. Surprisingly, although administration of L-NMMA to mice had no significant effect on bone marrow cellularity, simultaneous treatment of mice with benzene resulted in a dose-dependent decrease in bone marrow cell number (*3*), suggesting that L-NMMA potentiates benzene-induced hematotoxicity. To further characterize the effects of L-NMMA on bone marrow cells, we analyzed nitric oxide production. Unexpectedly, we found that cells from mice treated with L-NMMA either alone, or in combination with benzene, produced greater amounts of nitric oxide in response to LPS or LPS plus GM-CSF than did cells from control mice (Figure 3) (*23*). These data indicate that L-NMMA, like benzene, primes bone marrow cells to respond to growth factors and inflammatory mediators. Cells from mice treated with benzene and L-NMMA also produced more nitric oxide in response to LPS than did cells from animals treated with either of these agents alone (Figure 3) (*3,23*), a finding consistent with the decrease in bone marrow cellularity found in these mice.

Discussion

In the presence of inflammatory mediators such as LPS and IFN-γ, cells from the bone marrow have the capacity to produce nitric oxide. The colony-stimulating factors GM-CSF, M-CSF, and IL-3, although

inactive alone, enhance nitric oxide production induced by the inflammatory mediators. Interestingly, production of nitric oxide by bone marrow cells was inversely correlated with their proliferative capacity. Thus, cells that produce the greatest amounts of nitric oxide exhibit the lowest proliferative capacity. In excessive amounts, nitric oxide is cytotoxic (*19,20,28–31*). It has the capacity to abstract electrons from electron-rich substrates, including critical enzymes containing heme and iron–sulfur-containing proteins such as those involved in energy metabolism and DNA replication (*19,20*), and this may occur in bone marrow cells producing nitric oxide. We found that treatment of mice with benzene or its metabolites enhanced the responsiveness of bone marrow cells to inducers of nitric oxide. These data suggest that this free radical may play a role in regulating hematopoietic cell growth and maturation during benzene-induced bone marrow suppression.

At the present time, the mechanisms by which benzene and its metabolites enhance nitric oxide production in the bone marrow are unknown. In earlier studies we demonstrated enhanced production of interleukin-1 and TNF-α in bone marrow cells after benzene treatment of mice (*16*). These cytokines are potent activators of iNOS (*18,32*), and it is possible that these or other inflammatory cytokines act in an autocrine or paracrine manner to regulate nitric oxide production in bone marrow cells. In this regard, endogenous interferon-β, but not TNF-α, synergizes with suboptimal concentrations of LPS to activate inducible nitric oxide synthase (*33*). We have previously shown that pulmonary irritants upregulate expression of platelet-activating factor receptors on pulmonary alveolar macrophages, which is associated with increased responsiveness to this proinflammatory cytokine (*34*). It is possible that benzene and its metabolites upregulate expression of receptors for inflammatory mediators on bone marrow cells, thus increasing their sensitivity to these mediators.

If increased production of nitric oxide by bone marrow cells after benzene exposure contributes to hematotoxicity, then it might be expected that administration of the nitric oxide synthase inhibitor L-NMMA would ameliorate the toxic effects of this solvent. Unexpectedly, L-NMMA potentiated benzene-induced hematotoxicity (*3*). In addition, L-NMMA by itself caused an overall decrease in bone marrow cell proliferation. It also reduced the increased sensitivity of bone marrow cells from benzene-treated mice to the growth-promoting effects of GM-CSF. Taken together, these data indicate distinct mechanisms of action of L-NMMA and benzene. At the present time, the mechanisms underlying the toxic effects of L-NMMA are not known. Although one site of action is inhibition of nitric oxide synthase, L-NMMA, an arginine analog, may also interfere with other metabolic pathways utilizing this amino acid. In this regard, in many cell types, L-NMMA is known to inhibit arginine transport into cells (*35*), and limiting nutrient supply may lead to toxicity. Another possibility is that L-NMMA, like benzene, also functionally activates phagocytes in the bone marrow. Hematotoxicity could then result from an increase in the production of reactive mediators by these cells.

The precise role of nitric oxide in benzene-induced bone marrow toxicity remains to be elucidated. Nitric oxide has been implicated in the toxicity of a number of diverse xenobiotics including ozone, silica, paraquat, endotoxin and acetaminophen (*18*). With many of these agents, enhanced production of reactive oxygen intermediates in target cells has been reported (*18*). Interestingly, benzene treatment of mice also results in enhanced production of reactive oxygen intermediates by bone marrow phagocytes (*17*). Nitric oxide reacts rapidly with superoxide anion, forming peroxynitrite. This reaction may effectively decrease toxicity by reducing oxidant levels in the tissue; however, it may also augment tissue damage because peroxynitrite is a powerful oxidizing agent (*21*). In the presence of molecular oxygen, nitric oxide can also form a variety of nitrogen oxide intermediates that are potent *N*-nitrosating agents that can react with primary and secondary amines to form nitrosoamines (*36*). Formation of these highly carcinogenic metabolites may be responsible for the mutagenic activity of nitric oxide (*37,38*) and could account for the known leukemogenic activity of benzene. Further studies are in progress to explore these possibilities.

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