Subclinical Effects of Groundwater Contaminants I: Alteration of Humoral and Cellular Immunity by Benzene in CD-1 Mice

Gin C. Hsieh*, Raghubir P. Sharma**,1, and Robert D. R. Parker*

Toxicology Program, Departments of Biology* and Animal, Dairy & Veterinary Sciences,** Utah State University, Logan, Utah 84322

Abstract. Benzene is a known contaminant found in trace amounts in ground water. It has long been associated with myelotoxicity and associated immunologic effects. The present study concerned the immunotoxic potential of benzene following four weeks of continuous oral administration via drinking water at concentrations of 0, 31, 166 and 790 mg/L. Benzene-treated water produced a dose-related decrease in spleen weight and increase in kidney weight; both were significantly different at the highest level. Benzene exposure caused a significant dose-response reduction of peripheral blood leukocytes, lymphocytes, erythrocytes and resulted in a severe macrocytic anemia. Splenic lymphocyte proliferation to both B cell and T cell mitogens [lipopolysaccharide (LPS), pokeweed mitogen (PWM), concanavalin A (Con A) and phytohemagglutinin (PHA)] was followed by a dose-related biphasic responsiveness, enhanced at the lowest dose (31 mg/L) and depressed in the higher dosage groups (166 and 790 mg/L). Cell-mediated immunity as measured by mixed-lymphocyte culture (MLC) response to allogeneic cells and cytotoxic T lymphocyte (CTL) activity to YAC-1 tumor cells exhibited similar biphasic phenomenon. Antibody production as assessed by enumeration of the sheep red blood cell (SRBC)-specific plaque-forming cells (PFC) indicated a significant suppression of PFC in animals exposed to 166 and 790 mg/L benzene. A decrease in the α-SRBC-antibody titer corresponded to the numbers of PFC. The findings suggest that oral ingestion of benzene, at the concentrations utilized, produced a biologically significant immunotoxic effect on both the humoral and cellular immune responses.

Concern for the hazards of pollutants present in ground water is attracting attention, since ground water is a major source of drinking water supplies. The major concern is the possible subclinical effects after long-term exposure. The immune system as a target organ for detecting toxicity is being studied, since its sensitive parameters are easily evaluated (Bick et al. 1985; Dean et al. 1982). A number of industrial and environmental chemicals induce immunosuppression (Faith et al. 1980). Benzene, a ubiquitous environmental pollutant, exerts its greatest toxicity on hematopoietic cells, including erythroid, myeloid and lymphoid lineages, both in the peripheral blood and in the hematopoietic organs (Fishbein 1984; Bolcsak and Nerland 1983). Repeated benzene exposure in humans and laboratory animals induces various forms of cytopenia, aplastic anemia, leukemia and the development of chromosomal abnormalities (Dean 1985; Green et al. 1981; USEPA 1980; Brief et al. 1980). The mechanism for benzene-induced hematopoietic effects has not been well elucidated; however, most researchers believe oxidative benzene metabolites, rather than benzene itself, are required to mediate these hematopoietic effects of benzene (Sawahata et al. 1985; Irons 1985; Bolcsak and Nerland 1983; Greenlee et al. 1981).

Lymphocytes in animals are sensitive to benzene toxicity (Rozen and Snyder 1985; Dempster et al. 1984; Snyder et al. 1982; Green et al. 1981; Aoyama 1986), as are those from humans who are inadver-

1 Address correspondence to R. P. Sharma, Utah State University, Logan, Utah 84322-5600.
tently exposed to this chemical (Moszczynsky and Lisiewicz 1984; Brief et al. 1980). Benzene-induced depressions in lymphocyte counts may impair immunocompetence, since lymphocytes play a principal role in immune functions. The short-term administration of benzene by inhalation or intraperitoneal injection to experimental animals has caused abnormalities of immune-associated parameters, including a suppression in mitogenic response of B- and T-lymphocyte proliferations (Rozen and Snyder 1985; Rozen et al. 1984; Wierda et al. 1981; Aoyama 1986), impaired humoral antibody response as measured by plaque-forming cells to sheep erythrocytes (Wierda et al. 1981 Aoyama 1986), and increased susceptibility to the pathogenic microorganism Listeria monocytogenes (Rosenthal and Snyder 1985; Aranyi et al. 1986). Similarly, treatment with benzene metabolites has also impaired the immune responses (Pfefer and Irons 1982; Wierda and Irons 1982).

The inbred C-57BL mice used in experiments described above may harbor an endogenous lymphoma virus (Kaplan 1967); activation of this latent virus after benzene treatment may influence the toxicity of benzene (Snyder et al. 1980; Longacre et al. 1981). The outbred CD-1 mice were used in the current study. There are relatively few reports on effects of benzene after oral ingestion. Because some water supplies may be contaminated with benzene (USEPA 1980), oral administration was employed via drinking water for a 4-week period.

**Materials and Methods**

**Animals**

Male, and adult CD-1 mice (Charles River Breeding Laboratory Inc., Wilmington, MA) were procured at 5 to 6 weeks of age (approximately 18 g) and were acclimatized to the animal care facility for one week before use. Mice were randomly assigned to control and treatment groups and housed five per group in plastic cages with hardwood-chip bedding. They were maintained on laboratory rodent chow and tap water ad libitum. Room conditions were kept at an ambient temperature of 21 ± 1°C and a relative humidity of 50 ± 10%. The light/dark cycle was maintained at 12-hr intervals.

**Exposure**

Analytical reagent grade benzene (99.9% purity, JT Baker Chemical Co., Phillipsburg, NJ), was dissolved in normal tap water to make the intended levels of 40, 200, and 1,000 mg/L. Benzene has a solubility of 1,780 mg/L in water at 25°C (USEPA 1980). The benzene-treated water was administered to mice continuously for 28 days via drinking water; the control group received untreated tap water. To minimize decomposition and to maintain the concentration of benzene, drinking water was provided in glass water bottles, shaken frequently during treatment and was changed every 3 days. Feed and water consumption was monitored continuously, and animals were weighed once each week. Benzene concentration in drinking water was confirmed on different days by gas chromatography (USEPA 1982).

**Gross Observations and Hematology**

Twenty-eight days after exposure to benzene, mice were killed by decapitation, and gross pathological examinations were performed on all mice. Major organs, i.e., liver, spleen, thymus, and kidney were removed, trimmed and weighed. Blood samples were collected in siliconized test tubes coated with potassium ethylene-diaminetetraacetate. Leukocytes and erythrocytes were counted with an automated electronic cell counter (Model ZBI, Coulter Electronics, Inc, Hialeah, FL). Hematocrits were performed with microhematocrit equipment. Differential leukocyte counts were evaluated by Wright’s-Giemsa stained smears.

**Isolation and Culture of Splenic Lymphocytes**

After sacrifice, the spleen was aseptically removed from each animal, rinsed in icecold sterile isotonic saline and single cell suspensions were prepared and cultured according to Sharma and Gehring (1979). Cells were suspended in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (Hyclone Sterile Systems, Inc, Logan, UT) and 100 units penicillin and 100 μL streptomycin per mL. Cell counts were made with a Coulter counter.

**Lymphocyte Proliferation to Mitogens**

Splenic lymphocytes were plated in triplicate cultures (5 × 10⁶ cells/culture well) in 96-well flat-bottom microtiter plates (Microtest II, Falcon Plastics, Oxnard, CA) to assay their responses to E. coli lipopolysaccharide (LPS; Sigma Chemical Co, St. Louis, MO), pokeweed mitogen (PWM; Gibco Laboratories, Grand Island, NJ), concanavalin A (Con A; Sigma Chemical, St. Louis, MO), or phytohemagglutinin (PHA; Wellcome Reagents Ltd, Beckenham, England). Culture media with or without mitogen was added, total well volume was adjusted to 0.15 mL. The optimized concentrations of mitogens were 20 μg/mL, 30 μg/mL, 2.5 μg/mL, and 6.0 μg/mL for LPS, PWM, Con A and PHA, respectively. Cultures were incubated at 37°C in a humidified atmosphere with 3.5–4.0% CO₂ for 48 hr and harvested after a 6-hr pulse with 0.5 μCi of [methyl-3H]-thymidine (H-TdR; New England Nuclear, Boston, MA) per well. The cells were collected on a glass fiber filter using an automated multiple sample cell harvester (Model M12, Biomedical Research and Development Laboratory, Rockville, MD). The amount of radioactivity (dpm) incorporated was measured with a scintillation counter (Model 2660, Packard Instrument Co, Downers Grove, IL).