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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-I 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 (Pfeifer 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 $21 \pm 1^\circ\text{C}$ and a relative humidity of $50 \pm 10\%$. 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 re-

ceived 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 (5×10^5 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 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, 2.5 $\mu\text{g/mL}$, and 6.0 $\mu\text{g/mL}$ for LPS, PWM, Con A and PHA, respectively. Cultures were incubated at 37°C in a humidified atmosphere with 3.5–4.0% CO_2 for 48 hr and harvested after a 6-hr pulse with 0.5 μCi of [methyl- ^3H]-thymidine (^3H -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).

Mixed Lymphocyte Culture (MLC) Responses

The proliferation of splenic lymphocytes (responders) from benzene-treated or control animals in response to challenge with allogeneic YAC-I tumor cells (stimulators) was evaluated. The exponential growing phase YAC-I cells previously maintained in culture medium were treated with 50 $\mu\text{g}/\text{mL}$ mitomycin C (Sigma) for 1 hr at 37°C, then washed 3 times immediately before putting into culture. Cultures were set up in triplicate in 96-well microtiter plates. Responder cells from the spleen of CD-1 mice were cocultured alone or with stimulator cells at a responder-stimulator ratio of 2:1. The proliferative response was assessed by [^3H]-thymidine incorporation during the last 24 hr of a 3-day culture. The cultures were harvested and counted as described for the mitogenesis assays above.

Cytotoxic T-Lymphocyte (CTL) Responses

Cytotoxic T-lymphocyte (CTL) assay was performed as described by Grabstein (1980). The splenic lymphocytes (responder cells) were incubated with mitomycin C-treated YAC-I cells (stimulator cells) for 5 days at 37°C in a 3.5–4.0% CO_2 atmosphere. These incubated splenic cells (effector cells) were cocultured in triplicate with ^{51}Cr -labeled YAC-I cells (target cells) in a total volume of 200 μL in the microtiter plate. The desired effector-to-target cell ratios were 50:1 and 25:1. Effector and target mixtures were spun for 1 min at 1000 g, and then incubated for another 4 hr at 37°C. After incubation, cells were pelleted by centrifugation for 10 min at 1000 g, and the supernatants were decanted and counted in a gamma counter (Model 3320, Packard). The labeled target cells in the presence of complete medium or 0.5% sterilized saponin served as the spontaneous ^{51}Cr released and total ^{51}Cr released, respectively. The triplicate mean was used to calculate the percentage of specific lysis of target cells as described earlier (Grabstein 1980).

Plaque Forming Assay

Numbers of IgM plaque-forming cells (PFC) were determined by the method of Jerne and Nordin (1963) as modified by Cunningham and Szenberg (1968). Each mouse from control and test groups was sensitized by i.p. injection with 0.25 mL of a 20% sheep red blood cell (SRBC) suspension 4 days before the end of the benzene exposure. Splenic cell suspensions were prepared (1×10^7 cells/mL) as described above. A mixture containing 45% splenic cells suspension, 45% of a 10% SRBC suspension, and 10% guinea pig complement (Cappel Laboratories, Cochranville, PA) was delivered to counting chambers, three replicates per animal. Cultures were incubated at 37°C for 1 hr and PFC were calculated and expressed as plaques/ 10^6 splenic cells or plaques/total splenic cells.

Enzyme-linked Immunosorbent Assay (ELISA)

Double sandwich enzyme-linked immunosorbent assay (ELISA) was used to analyze anti-SRBC (α -SRBC) antibodies (Voller *et al.* 1979). Serum samples were collected from mice utilized in the plaque-forming assay. Plates (Gilford polystyrene EIA cu-

vettes, Medical Instruments Laboratories, Inc, Oberlin, OH) were coated with 200 μL goat red blood cell ghosts (prepared as described by Hannhan and Ekholm 1974) containing 3.6 $\mu\text{g}/\text{mL}$ protein solubilized in 0.1% sodium dodecyl sulfate, and kept at 4°C for 24 hr. After several rinses, 200 μL aliquots of sera (dilution 1:150) were added to the plate wells and incubated for 2 hr at 37°C and rinsed. Subsequently, 250 μL peroxidase-conjugated goat anti-mouse immunoglobulins (Hyclone Sterile Systems, Inc, Logan, UT) diluted 1:2000 were pipetted to each well to determine α -SRBC antibody levels, and incubated another 3 hr at 37°C. The enzyme reaction was performed by adding the phosphate-citrate buffered substrate solution containing 0.4 mg/mL *o*-phenylenediamine (Sigma) and 0.4 $\mu\text{L}/\text{mL}$ H_2O_2 , prepared immediately before use. The results were expressed as change in absorbance (ΔOD) 15 min after the addition of substrate solution. Non-sensitized mice pooled sera were used as a negative control. The optical density was determined by an automated spectrophotometer (Gilford, Model EIA 50, Oberlin, OH); readings were made at 490 nm.

Statistical Analysis

The results are expressed as the means and their standard errors. Statistical significances for differences between benzene-exposure and corresponding normal water control values were determined by a one-way analysis of variance (ANOVA) design followed by multiple comparison procedures of Fisher's least significant differences (LSD) test (Dowdy and Wearden 1983), when F ratios indicated significant difference. A level of $p < 0.05$ was considered statistically significant.

Results

The average concentration of benzene in drinking water as determined by gas chromatographic analysis on several days is indicated in Table I. There was a 10% loss of benzene on the first day, an additional 10% loss per day followed on second and third day, when the water was changed. Based on observed average intake of benzene-treated water, the daily dose of benzene in each animal was estimated to be 0, 8, 40 and 180 mg/kg, respectively.

Body and Organ Weights

Continuous exposure of adult, CD-1 male mice to various levels of benzene via drinking water over a 4-week period did not produce overt clinical symptoms of toxicity. Overall, there was no change in food and water consumption. No gross lesions were observed on any of the organs of mice in all treatment groups. There was no apparent alteration in body weight gain at termination of the study. Although the two groups receiving higher doses (40 and 180 mg/kg/day) had a slight increase in growth

Table 1. Organ and body weights of mice following 4 weeks of benzene exposure^a

Concentration in water, mg/L		Dose mg/kg/day	Body weight (g) ^b		Organ weight (g/100 g body weight) ^b			
Nominal	Observed ^c		Day 0	Day 28	Spleen	Liver	Kidney	Thymus
0	0	Control	23.20 ± 0.37	31.22 ± 0.62	0.33 ± 0.02	5.57 ± 0.13	1.55 ± 0.06	0.16 ± 0.02
40	31	8	22.68 ± 0.33	29.94 ± 0.98	0.30 ± 0.01	5.46 ± 0.29	1.58 ± 0.08	0.12 ± 0.01
200	166	40	23.74 ± 0.22	32.98 ± 0.65	0.28 ± 0.01	5.86 ± 0.07	1.65 ± 0.10	0.12 ± 0.01
1000	790	180	23.14 ± 0.29	32.26 ± 0.86	0.26 ± 0.01*	5.98 ± 0.20	1.87 ± 0.05*	0.11 ± 0.01

^a Benzene was administered continuously via drinking water for 4 weeks^b Values are given as mean ± SE (n = 5)^c The observed overall ranges of benzene concentration were 28 to 36, 147 to 178, and 734 to 855, in the three treatment groups, respectively

* Significantly (p < 0.05) different from control values

Table 2. Effects of benzene exposure on selected blood parameters

Dose ^a mg/kg/day	Erythrocytes (10 ⁶ /mm ³)	Hematocrit (%)	MCV ^b (fL)	Leukocytes (10 ³ /mm ³)	Leukocyte absolute differentials		
					Lymphocytes (10 ³ /mm ³)	Neutrophils (10 ³ /mm ³)	Others ^c (10 ³ /mm ³)
Control	7.01 ± 0.65 ^d	50.30 ± 0.58	74.01 ± 6.03	5.96 ± 0.60	4.38 ± 0.52	1.10 ± 0.13	0.45 ± 0.08
8	4.57 ± 0.28*	48.80 ± 0.46	108.30 ± 6.47*	4.23 ± 0.07*	2.99 ± 0.07*	0.85 ± 0.10	0.38 ± 0.05
40	3.68 ± 0.65*	46.20 ± 0.46*	125.18 ± 15.68*	4.22 ± 0.57*	2.35 ± 0.30*	1.46 ± 0.24	0.40 ± 0.09
180	3.42 ± 0.39*	45.00 ± 1.22*	136.76 ± 12.57*	2.87 ± 0.16*	1.61 ± 0.10*	1.02 ± 0.07	0.25 ± 0.09

^a Benzene was administered continuously via drinking water for 4 weeks^b Mean corpuscular volume^c Including monocytes, eosinophils and basophils^d Values are given as mean ± SE (n = 5)

* Significantly (p < 0.05) different from control values

rate, growth was not significantly different than the controls (Table 1). The weights of selected organs are also presented in Table 1. Weights of spleen, a secondary lymphatic tissue, decreased in a dose-related manner. Kidney weights correspondingly increased. Thymus weight was reduced in all treated groups but not significantly.

Hematological Parameters

The effects of benzene exposure on erythrocyte counts, hematocrits, mean corpuscular volumes (MCV), leukocyte counts, and leukocyte differential counts are presented in Table 2. Erythrocytes decreased significantly as dose increased. Hematocrits were depressed significantly at the 40 and 180 mg/kg/day of benzene. However, all of the MCV values of treated animals, when calculated from the hematocrits and erythrocyte counts, exhibited a significant increase. There was a significant dose-related reduction in total circulating white cells

after oral ingestion of benzene. Lymphocyte population decreased significantly when leukocyte differentials were expressed on an absolute basis. The number of neutrophils and other white blood cells (monocytes, eosinophils and basophils) were not altered by the benzene exposure.

Mitogen-induced Lymphocytic Proliferations

Following four weeks of benzene treatment, the proliferative response of either mitogen-stimulated or nonstimulated splenic lymphocytes were elevated in low-dose groups and depressed in higher-dose groups (Table 3). At the optimal concentration of LPS (20 µg/mL), ³H-TdR uptake increased significantly in the group receiving 8 mg/kg/day of benzene. In contrast, the benzene-treated groups receiving 40 and 180 mg/kg/day manifested a significant suppression of ³H-TdR incorporation of splenocytes upon LPS stimulation. Similarly, there were significant biphasic alterations of lymphopro-

Table 3. Effect of benzene exposure on splenic lymphocyte proliferative response to mitogens

Dose ^a mg/kg/day	Spleen cellularity ^b ($\times 10^{-7}$)	Mitogen responses ^c				
		None	LPS	PWM	Con A	PHA
Control	7.51 $\pm$ 0.40 ^d	1.68 $\pm$ 0.31	102.04 $\pm$ 23.66	7.45 $\pm$ 1.90	154.69 $\pm$ 49.33	68.02 $\pm$ 18.57
8	7.91 $\pm$ 0.62	3.55 $\pm$ 0.89*	261.23 $\pm$ 25.62*	12.92 $\pm$ 2.19*	384.90 $\pm$ 108.61*	285.81 $\pm$ 88.01*
40	7.50 $\pm$ 0.41	1.13 $\pm$ 0.13	22.43 $\pm$ 3.48	3.25 $\pm$ 0.71	27.30 $\pm$ 7.69*	16.43 $\pm$ 3.94
180	5.17 $\pm$ 0.64*	0.91 $\pm$ 0.19	25.44 $\pm$ 6.20	3.65 $\pm$ 0.72	14.61 $\pm$ 2.66*	8.87 $\pm$ 1.83*

^a Benzene was administered continuously via drinking water for 4 weeks^b Recovered number of splenocytes per spleen $\times 10^{-7}$ ^c $\text{dpm} \times 10^{-3}/10^6$ splenic cells: response evaluated by incorporation of [methyl-³H]-thymidine into day 2 splenocyte cultures for 6 hr pulsing^d Values are given as mean $\pm$ SE (n = 5)

* Significantly (p < 0.05) different from control values

Table 4. Effect of benzene exposure on mixed lymphocyte culture response^a

Dose ^b mg/kg/day	DPM/ 10^6 cells ($\times 10^{-3}$)	
	Responders	Responders + Stimulators
Control	2.11 $\pm$ 0.30 ^c	31.49 $\pm$ 2.77
8	3.59 $\pm$ 0.87	56.24 $\pm$ 1.59*
40	1.71 $\pm$ 0.42	19.77 $\pm$ 2.36*
180	1.58 $\pm$ 0.18	9.30 $\pm$ 0.88*

^a Responder cells were the syngeneic splenic cells of CD-1 mice and stimulator cells were the allogeneic YAC-1 cells. A ratio of 1:2 stimulators to responder was used, and the stimulator cells were treated with mitomycin-C before adding to the culture. MLC was evaluated by incorporation of [methyl-³H]-thymidine into day 3 MLC cultures after 6 hr pulsing^b Benzene was administered continuously via drinking water for 4 weeks^c Values are given as mean $\pm$ SE (n = 5)

* Significantly (p < 0.05) different from control values

liferation in T cell mitogen-treated cells; both the Con A and PHA-induced response were enhanced in the lowest treatment group and inhibited at the two high levels. The PWM or nonmitogen-stimulated splenocytes increased significantly in the animals treated with the lowest dose of benzene but were insignificantly depressed in groups receiving higher amounts. Total spleen cellularity decreased significantly at the highest dose of benzene treatment.

MLC and CTL Responses to Allogeneic Cells

The results of MLC presented in Table 4 demonstrate a dose-related biphasic response, *i.e.*, ³H-TdR uptake was inhibited at the higher benzene doses but increased in the lowest dose group. The effect

Table 5. Effect of benzene exposure on cytotoxic T-lymphocyte response

Dose ^a mg/kg/day	% Cytotoxicity	
	50:1 ^b	25:1
Control	15.72 $\pm$ 1.16 ^c	15.98 $\pm$ 2.98
8	17.94 $\pm$ 1.61	23.32 $\pm$ 2.05*
40	8.77 $\pm$ 1.87*	11.09 $\pm$ 1.71
180	5.82 $\pm$ 0.94*	7.17 $\pm$ 1.53*

^a Benzene was administered continuously via drinking water for 4 weeks^b Effector (day 5 MLC lymphocytes)-to-target (⁵¹Cr labelled YAC-1 cells) cell ratio^c Values are given as mean $\pm$ SE (n = 5)

* Significantly (p < 0.05) different from control values

of benzene exposure on CTL activity is shown in Table 5. The results of ⁵¹Cr-release assay revealed that benzene significantly impaired the capacity of cytotoxic T-lymphocytes to lyse alloantigenic cells (target cells) at 25:1 and 50:1 effector (E)-to-target (T) cell ratios after 4 weeks of oral ingestion. The lowest dose (8 mg/kg/day) of benzene obviously enhanced the CTL responsiveness in 25:1 E:T ratio.

Antibody Production

The primary antibody response to SRBC was assessed four days later after sensitization with SRBC; the SRBC-specific plaque-forming cells (PFC) were counted, and the α -SRBC antibody titer determined. As shown in Table 6, ability of treated animals to produce SRBC-specific antibody changed. The number of PFC in the animals receiving 40 and 180 mg benzene/kg/day were significantly reduced, when expressed on either specific activity (PFC/ 10^6 spleen cells) or whole spleen

Table 6. Effects of four weeks of benzene exposure on the antibody responses to thymic-dependent antigen sheep erythrocyte (SRBC)^a

Dose ^b mg/kg/day	Spleen cellularity ($\times 10^{-7}$)	PFC/10 ⁶ Splenic cells	PFC/total spleen cells ($\times 10^{-3}$)	α -SRBC titer
Control	22.59 $\pm$ 3.02 ^c	1.254 $\pm$ 171	295.72 $\pm$ 74.51	0.44 $\pm$ 0.06
8	16.80 $\pm$ 1.59*	1,576 $\pm$ 65*	268.80 $\pm$ 36.10	0.57 $\pm$ 0.11
40	15.36 $\pm$ 0.65*	643 $\pm$ 4Y*	Y9.76 $\pm$ 10.32*	0.31 $\pm$ 0.05
180	12.70 $\pm$ 1.51*	229 $\pm$ 40*	31.41 $\pm$ 9.52*	0.21 $\pm$ 0.01*

^a Mice were sensitized with SRBC 4 days before the end of the benzene exposure. Splenic lymphocytes were analyzed for antibody forming cells and sera were detected for antibody titer (α -SRBC)

^b Benzene was administered continuously via drinking water for 4 weeks

^c Values are given as mean $\pm$ SE (n = 5)

* Significantly ($p < 0.05$) different from control values

basis. However, there were more PFC per 10⁶ splenic cells in animals exposed to the lowest level of benzene. The total number of recovered splenocytes was significantly reduced in a dose-related manner. The titers of α -SRBC antibodies corresponded to the numbers of PFC, but significant depression was only found at the highest concentration of benzene (Table 6).

Discussion

The results demonstrated that oral administration of various dosages of benzene via drinking water *ad libitum* to CD-1 mice markedly altered the immune function. The alteration of immunocompetence was grossly evidenced by a decrease of thymic and splenic weights, which are useful indicators of immune dysfunction, and a loss of lymphocytes from peripheral blood and spleen. Benzene appeared to specifically affect lymphocytes since neutrophil and other leukocytes did not decrease significantly (Table 2). Various studies have revealed that lymphocytes are particularly sensitive to benzene toxicity via inhalation (Rozen and Snyder 1985; Dempster *et al.* 1984; Snyder *et al.* 1982; Green *et al.* 1981). Benzene increases the permeability of lymphocyte lysosomal membranes and releases lysosomal enzyme into the cytoplasm, resulting in a decrease in the number of lymphocytes (Moszczynsky and Lisiewicz 1984). Mice exposed to 300 ppm benzene via inhalation showed significant depressions in the numbers of B-lymphocytes (s-IgM+ cells) in bone marrow and spleen and in the numbers of T-lymphocytes (Thy 1,2+ cells) in thymus and spleen (Rozen and Snyder 1985). Similar effects on these two lymphocyte populations in blood and spleen were also reported in mice after 7 or 14-day inhalation exposure to 50 or 200 ppm benzene (Aoyama 1986). Occupational exposure to

benzene showed significantly decreased T-lymphocyte counts (Moszczynsky and Lisiewicz 1984). In our studies, mice exposed to benzene had significantly lower peripheral erythrocyte counts and hematocrit. The increase in mean corpuscular volume (MCV) indicates that benzene ingestion induced a macrocytic anemia. In humans, chronic benzene poisoning is most easily detected by RBC reduction and increased MCV, but an accurate diagnosis also requires the other hematologic parameters (Haley 1977). Baarson *et al.* (1984) reported that repeated exposure to low levels of benzene (10 ppm) via inhalation in mice depressed the *in vitro* colony-forming ability of one of the erythroid progenitor cells, the colony-forming unit-erythroid (CFU-E), the number of splenic nucleated red cells, and the number of circulating erythrocytes and lymphocytes.

Generally, the size of lymphatic organs associated with immunologic function decreased. The effect of benzene on immune parameters in the present study was often a biphasic response, enhanced at the lowest dose (8 mg/kg/day) and suppressed at the higher doses (40 or 180 mg/kg/day). Several workers have reported the *in vivo* immunosuppressive effect of benzene (Aoyama 1986; Rozen and Snyder 1985; Pandya *et al.* 1986). However, differences in animal models, doses and duration of exposure, and routes of administration make comparisons difficult (Longacre *et al.* 1981). Inhalation of benzene depressed host resistance to the infectious agents, *Listeria monocytogenes* and *Klebsiella pneumoniae* (Rosenthal and Snyder 1985; Aranyi 1986). Mitogen-induced lymphoproliferation was suppressed in mice treated with benzene metabolites, *i.e.*, hydroquinone and catechol (Wierda and Irons 1982). The cell renewal rate as well as the rate of DNA, RNA, and protein synthesis in cultured leukocytes and lymphoma cell lines exposed to benzene or its metabolites were inhibited (USEPA 1980; Walker *et al.* 1985; Post *et al.*

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1985; Schwartz *et al.* 1985). Hemopoiesis *in vitro* in stromal cells pretreated with low doses of oxidative metabolites of benzene, such as hydroquinone, was enhanced (Gaido and Wierda 1984). Garnett *et al.* (1983) also obtained biphasic responses *in vitro* with CFUs (colony forming units-spleen) from mice that inhaled benzene. These results support the hypothesis that benzene or its metabolites may promote as well as suppress the hemopoietic system (Harigaya *et al.* 1981).

Recently, Post *et al.* (1985) indicated that exposure of T-lymphocytes to p-benzoquinone, one of the metabolites of benzene, inhibited the formation of lymphokine interleukin 2 (IL2). IL2, produced by T lymphocytes, is an important regulatory cytokine in lymphocyte proliferation since it is a growth factor for helper, and cytotoxic T lymphocytes (Dinarello and Mier 1986).

The data described in this report show that ingestion of benzene via drinking water in CD-1 mice noticeably affected all aspects of the immune system, including cellular and humoral parameters. Of particular importance was the fact that the effects were evident at the lowest dose of treatment and a no-effect level could not be established. Further studies involving lower levels of exposure are necessary to fully ascertain the safety of this chemical in groundwater.

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