

Inhaled Benzene Reduces Aspects of Cell-Mediated Tumor Surveillance in Mice

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Inhaled Benzene Reduces Aspects of Cell-Mediated Tumor Surveillance in Mice. ROSENTHAL, G. J., AND SNYDER, C. A. (1987). *Toxicol. Appl. Pharmacol.* 88, 35-43. Benzene is a potent bone marrow toxicant with particular activity against lymphocytes. Despite the recognized effects of benzene on lymphocyte populations, few data exist concerning the effects of benzene on *in vivo* immune responses. We have been conducting a series of studies concerning the effects of inhaled benzene on murine cell-mediated immune responses. The studies in this report involve the interaction of inhaled benzene with some of those cell-mediated immune responses associated with tumor surveillance. Exposures to 100 ppm benzene (5 days/week $\times$ 20 weeks) induced lethal tumor growth in 9/10 C57Bl/6 mice inoculated with 10^4 viable PYB6 tumor cells. Lethal tumor incidences in air controls and mice exposed to lower benzene concentrations were 3/10 or less. Exposures to 100 ppm benzene (5 days/week $\times$ 4 weeks) also reduced the tumor lytic abilities of cytotoxic T lymphocytes as determined by ^{51}Cr -release assays. In addition, splenocytes taken from mice exposed to 10 or 100 ppm benzene (5 days/week $\times$ 4 weeks) exhibited delays in peak mixed leukocyte responses. Coculture experiments demonstrated that these delays were not due to an induction of suppressor cell activity by benzene. There were no alterations in the relative percentages of B cells, T cells, or T-cell subsets among splenocytes from animals exposed to any concentration of benzene tested. These results demonstrate that inhaled benzene can inhibit some of the processes associated with tumor surveillance, and that this inhibition is due, at least in part, to impairments of the functional abilities of some of the cells responsible for tumor surveillance. © 1987 Academic Press, Inc.

Benzene is a well-known hematotoxicant (Goldstein, 1977; Aksoy, 1981). In humans, the predominant hemopathy associated with benzene exposure is pancytopenia or one of its variants (Goldstein, 1977). In animal studies, especially those employing rodents, pancytopenia is also a consistent finding with circulating lymphocytes showing particular sensitivity (Leong, 1977; Snyder *et al.*, 1978, 1980, 1982).

The sensitivity of lymphocytes to benzene indicates an immunotoxic potential for this

compound. Indeed, the results of a number of recent studies have implicated the immune system as a target for benzene. For example, benzene or known metabolites of benzene have been shown to suppress *in vitro* lymphoproliferative responses to mitogens (Wierda *et al.*, 1981; Pfeifer and Irons, 1981, 1982; Wierda and Irons, 1982). Although these studies further demonstrate lymphocyte sensitivity to benzene, they only bear inferentially on the possible interactions of benzene with the immune response.

We have been conducting a series of studies investigating the interactions of benzene with the murine immune response. In these studies, we have employed whole-body inha-

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lation exposures in order to address questions pertinent to occupational health. Earlier work from this laboratory showed that short-term inhalation exposures to low concentrations of benzene suppressed the mitogenic responses of B and T lymphocytes (Rozen *et al.*, 1984). More recently, we reported that inhalation of benzene altered the murine cell-mediated immune response to sublethal challenges by *Listeria monocytogenes* (Rosenthal and Snyder, 1985). We now report on further interactions of inhaled benzene with murine cell-mediated immune responses, specifically some of those associated with tumor surveillance.

METHODS

Animals. Male, 6-week-old C57Bl/6J mice were received from Jackson Laboratories (Bar Harbor, ME). Animals were quarantined for 2 weeks during which time they were acclimatized to a 12-hr light/dark cycle and observed for anomalous behavior and disease. Animals were randomly distributed into groups of 10 mice each for the tumor cell challenge study and 5 mice per group for the lymphocyte functional assays.

Inhalation exposure regimens. Animals were exposed in whole-body, 1.0-m³ dynamic inhalation chambers. During exposure periods, animals were housed in wire exposure cages. Benzene atmospheres were generated as previously described (Snyder *et al.*, 1978). Briefly, a stream of conditioned air was passed over a reservoir of benzene (chromatography grade, Alltech Associates, Ann Arbor, MI). The benzene-laden air was then delivered to the chamber supply air. Benzene concentrations were controlled by metering the flow rate of air that passed over the liquid benzene. Benzene concentrations of 30 and 100 ppm were monitored at 0.5-hr intervals during the exposures by means of a Miran 1A infrared spectrophotometer (Foxboro Analytical, South Norwalk, CT) at a wavelength of 9.8 μ m. Concentrations of 10 ppm were monitored at 0.5-hr intervals by bubbling a known volume of chamber air through ethanol and comparing the uv absorbance at 254.5 nm of the resultant benzene-ethanol solution with a previously prepared calibration curve. Chamber temperature and humidity conditions were also recorded at 0.5-hr intervals. Matched air control mice received the same exposure regimens but to conditioned air only. Exposures were conducted for 6 hr/day to either benzene or conditioned air.

For the tumor challenge experiments, groups of mice were exposed to either air, 10 ppm benzene, 30 ppm benzene, or 100 ppm benzene for 10 days (5 days/week). Fol-

lowing the 10th exposure, animals were inoculated with viable PYB6 tumor cells. After inoculation, the animals continued their respective air or benzene exposures until a total of 100 exposure days had been completed (5 days/week).

For the lymphocyte functional assays, groups of five animals were exposed to either air, or 10 ppm, 30 ppm, or 100 ppm benzene for 20 days (5 days/week).

Tumor challenge. A polyoma virus-induced tumor developed in C57Bl/6 mice (PYB6) was kindly provided by Dr. Michael I. Luster (NIEHS, Research Triangle Park, NC) and maintained by passage through syngeneic male C57Bl/6 mice. Two fresh tumors were taken from passage mice and minced in suspensions of complete medium (RPMI 1640 medium containing 25 mM Hepes buffer plus 10% fetal calf serum (GIBCO, Grand Island, NY)). Following preparation of a single-cell suspension and viability testing (trypan blue exclusion), mice were inoculated subcutaneously in the right rear flank with either 5×10^3 or 1×10^4 viable tumor cells suspended in RPMI 1640 medium. In our hands, these inocula caused a lethal tumor incidence of 10–30% in normal C57Bl/6 mice. Animals were palpated weekly and tumor frequency as well as latency (time to tumor development) were recorded. Tumor incidences were evaluated for differences using the log rank χ^2 method (Peto and Pike, 1973) which determines tumor incidence while correcting for mortality.

Splenic cell differential. Spleens were disrupted by forcing them through wire-mesh sieves into 10 ml of RPMI 1640 supplemented with gentamycin (50 μ g/ml), 10% fetal bovine serum, and 0.1% sodium azide. A single-cell suspension was achieved by gentle aspiration with a 3-cc syringe fitted with a 25-gauge needle. The cell suspensions were pooled from five mice in each exposure group and centrifuged at 200g for 10 min. The cell pellet was then exposed to 1 ml of 0.84% ammonium chloride solution for 10 min to lyse red blood cells. Cold medium (10 ml) was then added to the tubes and cells were washed twice. Cell concentrations were then determined by hemocytometer counting. Viability, measured by trypan blue exclusion, was between 86 and 93% in these preparations.

T and B lymphocytes were enumerated by a direct immunofluorescent technique described in the *Monoclonal Antibody Source Book* (Becton-Dickinson, 1981) using fluorescein isothiocyanate (FITC)-conjugated antibody. For T lymphocytes, the antibody used was monoclonally derived anti-Thy-1.2 (Becton-Dickinson, Mountain View, CA). For B lymphocytes, the antibody used was monoclonally derived anti-mouse IgM + IgG (Tago, Mountain View, CA). Working dilutions of 1:50 and 1:70 were used for Thy-1.2 and IgM + IgG antibodies, respectively, after preliminary studies with these dilutions gave results comparable to the published percentages for these cell types in normal C57Bl/6J mice. One million cells in 50 μ l of medium were combined with 50 μ l of antibody in a flat-bottomed microtiter plate. Cells

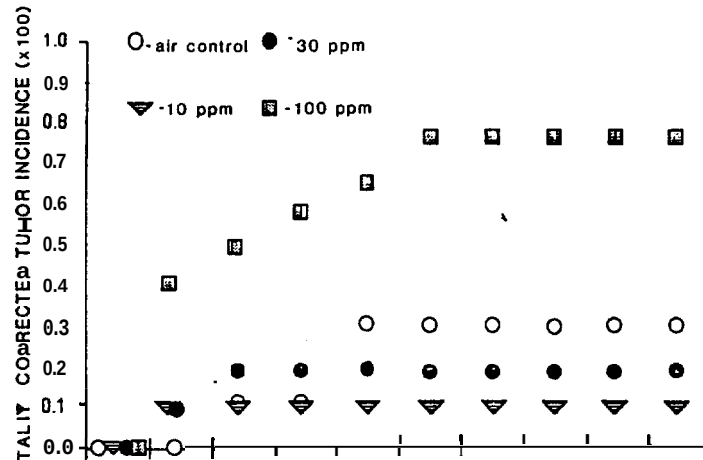


FIG. 1. Mortality-corrected plot of tumor incidence vs time; 10^4 PYB6 tumor cell inoculum. Data evaluated are from 10 animals per exposure concentration.

mixed with antibody were incubated on ice for 30 min and then washed twice with medium. The washed pellet was then resuspended in 25 μ l of Fluoromount-G (Southern Biotechnology, Atlanta, GA) and the smears enumerated for Thy-1.2-positive or IgM⁺IgG-positive cells by immunofluorescent microscopy using a Zeiss microscope with an attached uv source and appropriate filters. Between 200 and 400 cells on each of three slides were counted within 24 hr of smear preparation.

Monocyte/macrophages, polymorphonuclear leukocytes, lymphocytes, and nucleated red blood cells were scored from thin smears of nucleated spleen cells suspended in fetal calf serum. The smears were stained for nonspecific esterase (Koski *et al.*, 1976) and counterstained with Giemsa.

T-cell subset enumeration. T-cell subsets were enumerated using a cytotoxicity technique. Spleen cell suspensions pooled from five mice in each group were prepared as previously described with two modifications: all cellular handling and incubations were carried out in RPMI 1640 with 25 mM Hepes buffer and 0.3% bovine serum albumin (BSA, Cedarlane Cytotoxicity Medium, Cedarlane Labs, Ontario, Canada); and it was necessary to remove red cells and dead cells by purification of viable lymphocytes on Lymphocyte-M cell separation medium. Purification was carried out by layering 5 ml of the splenic cell suspension over 5 ml of Lymphocyte-M (Ficoll 400 and sodium diatrizoate; $d = 1.0875 \pm 0.0005$ at 25°C). The suspension was centrifuged for 20 min at 500g at room temperature, a well-defined lymphocyte layer was removed at the interface, and these cells were washed two or three times in medium before further processing. Cell concentrations were adjusted to 1×10^6 /ml in medium and five 1-ml aliquots were placed into five 12 $\times$ 100-mm polystyrene culture tubes. Either monoclonal anti-Lyt-1.2 or monoclonal anti-Lyt-2.2 (Cedarlane

Labs) or both antibodies were added in final concentrations of 1:30 and 1:1000, respectively, and incubated for 60 min at 4°C. These antibody concentrations were shown in preliminary studies to yield results comparable to the published percentages for these cell types in normal C57Bl/6 mice. Following incubation, cells were centrifuged once at 200g and the supernate was discarded. Cells were resuspended to original volume in Low-Tox-M rabbit complement (Cedarlane Labs) and this suspension was incubated for 60 min at 37°C. Cells were then placed on ice and trypan blue was added 3–5 min before scoring for dead cells in a hemocytometer. Controls included (1) cells alone, (2) cells with antibody alone, and (3) cells with Low-Tox rabbit complement alone. The numbers of lymphocytes in each subset were determined in three replicates from the spleen cells pooled from five mice in each exposure group. The percentages of Lyt subsets in the splenic cell population were calculated as $C = \text{total Lyt}^+$ population; $C - B = \text{Lyt-1 set}$; $C - A = \text{Lyt-2,3 set}$; $A + B - C = \text{Lyt-1,2,3 set}$ (Reske-Kunz *et al.*, 1979).

Antisera	Lyt set lysed			% Lysed
Lyt-1.2	Lyt-1	Lyt-1,2,3		A
Lyt-2.2		Lyt-2,3	Lyt-1,2,3	B
Lyt-1.2 + Lyt-2.2	Lyt-1	Lyt-2,3	Lyt-1,2,3	C

Mixed lymphocyte culture (MLC). Spleen cell suspensions from five individual mice were prepared as described above. Following one wash in RPMI 1640, total numbers of nucleated cells were determined with a Coulter counter (Model ZBI, Coulter Electronics, Hialeah, FL) and viability (always >90%) was assessed by trypan blue exclusion. Viable cells were resuspended at a concentration of 2×10^6 /ml of culture medium in sterile,

conical 15-ml polystyrene centrifuge tubes. The culture medium was RPMI 1640 supplemented with Hepes buffer (20 mM), L-glutamine (2 mM), 5% fetal calf serum (GIBCO), and 50 µg/ml gentamycin (Microbiological Associates, Walkersville, MD).

One-way mixed-leukocyte cultures were performed employing 5×10^5 mitomycin C (MMC; Sigma, St. Louis, MO)-treated cells from DBA/2 mice (stimulator cells) per well. MMC treatment was performed by incubating stimulator cells with MMC (50 µg/10⁷ cells/ml) for 40 min at 37°C. Following three washes in RPMI 1640, the stimulator cells were resuspended at 4×10^6 cells/ml in fresh RPMI 1640 medium with supplements to which was added 5×10^{-5} M 2-mercaptoethanol (Sigma). The stimulator cells and cells from either benzene-treated or air-exposed mice were added in 0.1-ml aliquots such that an effector to stimulator cell ratio of 1:2 was established (2×10^5 : 4×10^5) in each well. MLCs were harvested daily from the second through the eighth day of incubation following an 18-hr pulse with [³H]thymidine (1.0 µCi/well; sp act 5.0 Ci/mmol, Amersham, Northridge, IL). Cells were harvested using an automated cell harvester (PHD cell harvester, Cambridge Technology, Cambridge, MA) onto glass-fiber filters (grade 9H4 AHA). The filters containing radioactive material were placed in 7-ml glass scintillation vials containing 5 ml Aquasol scintillation fluid (Amersham), and the vials shaken vigorously. Following dark adaptation, the vials were counted for [³H]thymidine content using a LKB 1215 Rackbeta liquid scintillation counter (Wallac Oy Co., Turku, Finland).

Suppressor cell studies. Suppressor cell activity was examined by coculturing pooled MMC-blocked spleen cells (1×10^5 /well) from air or benzene-treated C57Bl/6J mice with spleen cells (1×10^5 /well) from untreated C57Bl/6J mice along with DBA/2 stimulator cells (4×10^5 /well, prepared as previously described) followed by examination of the mixed-leukocyte responsiveness at Days 2, 3, and 4 of culture. Counts were made on three replicates of pooled spleen cells from each group of five mice.

Cell-mediated cytotoxicity. Cytotoxic T-lymphocyte-mediated cytotoxicity was examined *in vitro* using splenocytes from benzene-exposed and air control animals as described by Brunner *et al.* (1968). Mice were exposed for 10 days to benzene or air and then sensitized by inoculation of P8 15 mastocytoma cells. Exposure to benzene or air were then continued for an additional 10 days. Spleen cells from five individual mice per group were tested for cytolytic activity using a ⁵¹Cr-release assay employing P8 15 cells as targets. P8 15 mastocytoma cells (H-2d haplotype) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained *in vitro*.

Tumor cells were collected from tissue cultures, washed three times in RPMI 1640, counted, and used to immunize mice at a concentration of 1×10^7 per mouse. Following a total of 20 exposures and 10 days of allogeneic

cell sensitization, animals were sacrificed and spleens removed. Splenic single cell suspensions were made as previously described in the MLC section. Target cells were labeled with 250 µCi of sodium chromate-51 (Amersham) by incubation at 37°C for 90 min under gentle agitation and then washed, counted, and resuspended at 2×10^5 cells/ml. A constant number of ⁵¹Cr-labeled target cells (2×10^4 in 100 µl) were added to each well of a plastic 96-well microtiter plate (Flow Laboratories, Bethesda, MD) followed by the addition of 100-µl aliquots of effector cells (splenic cells from air control or benzene-treated mice). Effector to target cell ratios were 100:1, 33:1, and 11:1. The 100:1 effector to target cell ratio was optimal for target cell lysis. Microtiter plates were incubated for 4 hr at 37°C. The plates were then centrifuged at 200g for 10 min at room temperature and 100 µl of supernatant fluid from each well was removed and counted in a gamma spectrophotometer. Counts were corrected for volume and compared to counts present in control wells containing target cells but no effector cells (spontaneous release). Maximum release of ⁵¹Cr from target cells was determined by addition of 50 µl of a 0.1% solution of Triton-X 100. Specific cytotoxicity was calculated as

$$\frac{\text{test release} - \text{spontaneous release}}{\text{maximum release}} \times 100.$$

Statistics. Analyses of variance were performed on the lymphocyte functional assay data. When *F* ratios indicated significant differences among the exposure groups, individual Student *t* tests were performed at a level of confidence such that the overall probability of a Type I error occurring was ≤ 0.05 . A two-tailed test with a combined *p* ≤ 0.05 level was considered significant.

RESULTS

Effects of Benzene Inhalation on Resistance to PYB6 Tumor Challenge

The mean daily benzene concentrations $\pm$ SD for the exposures were 101.4 ± 7.1 , 29.3 ± 2.1 , and 10.8 ± 1.5 ppm.

Following a total of 100 exposure days (5 days/week), the lethal tumor incidences for those mice receiving the 10^4 cell inoculum were 3/10, 1/10, 2/10, and 9/10 for air control, and 10, 30, and 100 ppm benzene, respectively. The lethal tumor incidences for those mice receiving the 5×10^3 cell inoculum were 2/10, 2/10, 3/10, and 3/10 for air control, and 10, 30, and 100 ppm benzene, respectively. A significant difference, as deter-

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TABLE 1

COMPARISON OF SPLENIC CELL POPULATIONS BETWEEN MICE EXPOSED TO 100 ppm BENZENE AND AIR CONTROLS

	Air control	100 ppm benzene
Splenic differential (%) ^a		
Lymphocytes	90.7 ± 1.5	88.7 ± 3.5
Thy-1.2 ⁺ cells	30.3 ± 3.1	33.0 ± 2.6
Ig ⁺ cells	56.3 ± 4.0	54.0 ± 2.6
Granulocytes	4.7 ± 2.1	6.3 ± 2.5
Esterase ⁺ cells	2.3 ± 1.5	3.0 ± 1.0
Splenic T-cell subsets (%) ^a		
Lyt-1 ⁺	44 ± 7	40 ± 6
Lyt-2,3 ⁺	9 ± 2	9 ± 4
Lyt-1,2,3 ⁺	48 ± 6	50 ± 9

^a Mean ± SD of triplicate determinations made from pooled ($n = 5$) spleen cell suspensions per treatment.

mined by log rank χ^2 analysis (Peto and Pike, 1973), was observed in the mice receiving the 10^4 cell inoculum and exposed to 100 ppm benzene. A plot of the data corrected for mortality shows this difference (Fig. 1). No differences in tumor incidences were observed among any groups inoculated with 5×10^3 tumor cells.

Lymphocyte Functional Assays

The mean daily benzene concentrations ± SD for the exposures used for the functional assays were 99.7 ± 7.0 , 29.5 ± 14.4 , and 11.1 ± 1.5 ppm.

Characteristics of Spleen Cell Populations following Benzene Inhalation

As shown in Table 1, twenty exposures to 100 ppm benzene induced no changes in the relative proportions of splenic leukocytes, in the percentages of splenic T-cell subsets, or in the ratio of splenic helper/suppressor cells (Lyt-1⁺/Lyt-2,3⁺).

Since the proportions of the various lymphocyte subpopulations were undisturbed, the lymphocyte functional assays described below could be normalized for particular lymphocyte populations by using equal numbers of splenic cells.

Kinetics of Mixed-Lymphocyte Culture following Benzene Inhalation

Assessments were made of the proliferative responses of spleen cells from air- or benzene-exposed mice in one-way mixed-lymphocyte cultures. Following 20 days of exposure of mice to either air, 10 ppm benzene, or 100 ppm benzene spleen cells were cultured and the amount of [³H]thymidine incorporated was assessed on Days 2 through 5 of culture. On Days 2, 3, and 4 of culture, the amounts of [³H]thymidine incorporated by cells from benzene-exposed mice were depressed relative to air control values (Fig. 2). On Day 5 of culture, however, the amounts of [³H]thymidine incorporated by spleen cells from benzene-exposed mice were increased over control values (Fig. 2). Thus, benzene exposure, even at 10 ppm induced a delay in the peak response of splenocytes to MMC-treated stimulator cells.

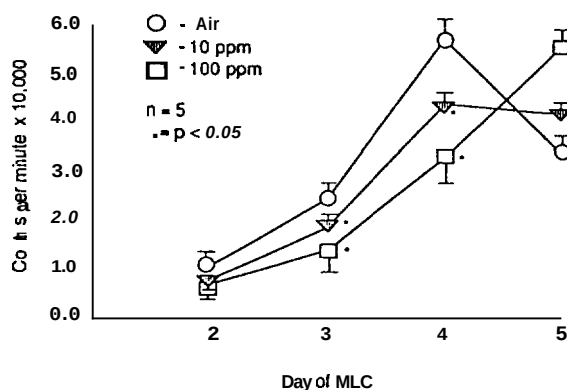


FIG. 2. Kinetic analysis of mixed-lymphocyte responses at Days 2 through 5 of culture following 20 days exposure to air or 10 or 100 ppm benzene. Individual functional tests were performed on cells isolated from each of five animals per exposure concentration. Error bars equal ± 1 SE. *Significantly different from air control.

TABLE 2
EFFECT OF 20-DAY EXPOSURE TO 100 ppm BENZENE ON POTENTIAL INDUCTION OF SUPPRESSOR
CELL ACTIVITY IN MIXED LEUKOCYTE CULTURE

Coculture	[³ H]Tdr incorporated (cpm)"		
	Day 2	Day 3	Day 4
1. Normal + stimulators (MMC) ^b	3020 ± 345	7,920 ± 873	15,685 ± 1278
2. Air (MMC) + stimulators (MMC)	1748 ± 40	1,822 ± 210	1,808 ± 208
3. Benzene (MMC) + stimulators (MMC)	1301 ± 308	1,310 ± 140	1,376 ± 121
4. Normal + stimulators (MMC) + benzene (MMC)	5570 ± 310	11,988 ± 1323	18,756 ± 756
5. Normal + stimulators (MMC) + air (MMC)	4125 ± 499	11,832 ± 752	17,249 ± 2279

^a Mean ± SD of triplicate determinations made from pooled (*n* = 5) spleen cell suspensions per treatment.

^b MMC = mitomycin C treated.

Suppressor Cell Studies

To determine whether benzene exposure could induce suppressor cell activity and thereby cause the depressed [³H]thymidine incorporation observed in the MLC at Days 2 through 4, a series of coculture experiments were performed. These coculture experiments were performed on Days 2, 3, and 4 of culture, when depressed [³H]thymidine incorporation was observed. Table 2 shows that coculturing of spleen cells from untreated C57Bl/6 mice with MMC-blocked spleen cells from either benzene-treated or air control mice induced no significant depression in [³H]thymidine incorporation. These data therefore suggest that the depressed responses observed at Days 2, 3, and 4 of the MLC were not due to benzene-induced suppressor cell activity.

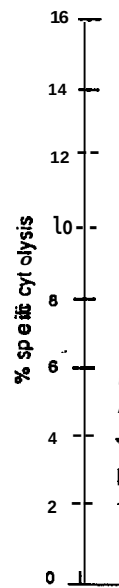
Cell-Mediated Cytolysis following Benzene Inhalation

The ability of sensitized cytotoxic T lymphocytes to recognize and destroy a sensitizing target cell was tested in a ⁵¹Cr-release cytotoxicity assay using spleen cells from mice

exposed to air or 10 or 100 ppm benzene for 20 days. Spontaneous release ± SD under experimental conditions employed was 723 ± 29 cpm. Total release ± SD of the 5 × 10⁴ ⁵¹Cr-labeled target cells was 3974 ± 196 cpm. The results of this experiment, presented in Fig. 3, demonstrate that exposure to 100 ppm benzene for 20 days significantly (*p* < 0.05) altered the ability of splenic lymphocytes to lyse the sensitizing alloantigen-bearing target cells at the effector to target (E/T) ratio of 100:1. No significant difference was observed between control and 10-ppm-benzene exposure groups at any of the E/T ratios tested (Fig. 3).

DISCUSSION

The results presented here demonstrate that inhaled benzene can cause immunodepressive effects as manifested by reduced host resistance to a transplantable syngeneic tumor and by altered *in vitro* lymphocyte functions. To our knowledge, this is the first study to investigate the effects of inhaled benzene exposure on host resistance to a tumor challenge and the results may bear on the known



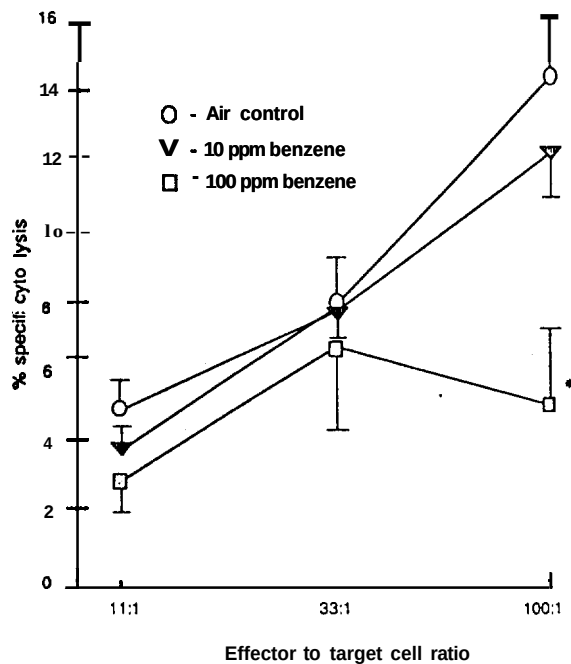


FIG. 3. Cytolytic activity of spleen cell population obtained from mice exposed for 20 days to air or 10 or 100 ppm benzene. Individual functional tests were performed on cells isolated from each of five animals per exposure concentration. Error bars equal ± 1 SE. *Significantly different from air control.

link between human exposure to benzene and hematopoietic neoplasms.

The tumor used in our host resistance study was a polyoma virus-induced tumor from C57Bl/6 mice (PYB6). There is a strong evidence that host resistance to this tumor and other DNA virus-induced tumors is mediated primarily via T lymphocytes (Dean *et al.*, 1975; Howell *et al.*, 1974; Law and Ting, 1965). Thus, these data suggest a benzene-induced impairment of T-cell-mediated immunity. However, in an *in vivo* situation, alterations of other effector cells (e.g., natural killer cells or macrophages) cannot be ruled out.

It is conceivable that a benzene-induced decrement in the number of immunocompetent cells caused the increased tumor susceptibility; however, using a ^{51}Cr -release assay we also demonstrated a benzene-induced impairment in the lytic ability of those T cells responsible for tumor cell lysis. In the ^{51}Cr -release assay we employed P815 mastocy-

toma cells which induce the production of cytotoxic T lymphocytes (CTL) (Brunner *et al.*, 1968). While an *in vitro* ^{51}Cr -release assay utilizing PYB6 cells as targets would correlate better with the tumor challenge data, generally syngeneic cells (as PYB6 are to C57Bl) do not elicit a marked CTL response *in vitro*. Although *in vitro* lysis of allogeneic P815 cells was employed in our studies, the mechanisms by which CTL destroy these cells *in vitro* is believed to be identical to the mechanisms by which CTL destroy virally infected or transformed cells *in vivo* (Wells, 1982).

In our studies the same benzene concentration (100 ppm) which altered host resistance to the PYB6 tumor challenge also impaired CTL cytotoxicity toward P815 mastocytoma cells. This further strengthens the association between a benzene-induced impairment of T-cell-mediated immune surveillance and increased susceptibility to the PYB6 tumor challenge.

The MLC was employed as a sensitive assay for the functional capacity of lymphocytes derived from animals exposed to benzene vapor. This assay is dependent upon cellular communication and T-cell recognition of antigen (F. H. Bach *et al.*, 1976; B. A. Bach *et al.*, 1978). The murine cell type widely thought to respond in the MLC is the Lyt-1^+23^- lymphocyte which is believed to lie within the same population of cells responsible for helper and delayed-type hypersensitivity reactions (Cantor and Boyse, 1975; Cantor *et al.*, 1976; Huber *et al.*, 1976). Cells from mice exposed to 100 and 10 ppm benzene showed increased [^3H]thymidine incorporation at Day 5 of the MLC, preceded by 3 days of depressed [^3H]thymidine incorporation (with respect to air controls). Thus, exposures to 10 ppm and exposures to 100 ppm altered the kinetics of [^3H]thymidine incorporation. Similar observations were reported by Rozen *et al.* (1984) in that 6 days of exposure to 10 ppm benzene was just as effective in reducing femoral B-cell blastogenesis as 6 days of exposure to 30, 100, or 300 ppm benzene. Apparently, concentrations lower than 10 ppm should be employed to ascertain concentra-

tion-response relationships between benzene exposures and these parameters.

It is noteworthy that **100** ppm benzene depressed responses in both the MLC and CTL assays. As MLC responsiveness is generally thought to represent the initial phases in the induction of cytotoxic T cells, the benzene-induced loss of CTL lytic ability might be explained by an impaired ability of T cells to recognize foreign tissue or tumor-associated antigens during T-helper-cell-mediated induction of cytotoxic T cells.

Alterations in immune function can be due, in part, to an induction of suppressor cell activity (Gershon, 1974; Berlinger *et al.*, 1976). Since the inhibitory effects of suppressor cells are exerted at the level of lymphocyte nucleotide synthesis (Kirchner *et al.*, 1975), a straightforward method for assessing suppressor activities is the *in vitro* coculture technique followed by [³H]thymidine labeling. A number of studies have shown that various agents can induce suppressor cell activity and thereby suppress *in vitro* lymphocyte function (Kirchner *et al.*, 1975; Clark *et al.*, 1983; Luster *et al.*, 1980). However, in our studies, excessive suppressor cell activity was not detected when coculturing experiments were performed in the context of the mixed-lymphocyte cultures (Table 2). The cultures for these experiments were harvested at Days 2 through 4 because it was at these time points that depressions were observed in the proliferative capacities of cells from mice exposed to **100** and **10** ppm benzene for 20 days.

Since the accessory functions of macrophages (e.g., antigen presentation, interleukin-1 secretion) are known to be required for most T-cell functions, we determined the effects of benzene exposure on the percentages of splenic monocytes and macrophages. Exposures to **100** ppm benzene for 20 days did not alter the normal percentages of these cells; however, these exposures may have affected the accessory functions of the macrophages and in this way contributed to the observed depressed T-cell functions.

The current time-weighted-average threshold limit value for benzene is **10** ppm with a

short-term exposure limit of 25 ppm (not to exceed **15** min over an **8** hr workday). Of the studies discussed above, the most pertinent to the threshold limit value was the delay in peak MLC response in mice exposed to **10** ppm. Under certain conditions, a delay in immune response might be detrimental to the host. For example, a delay in surveillance of tumor cells could enable the quantity of such cells to reach a number that would overwhelm the normal immune response. Since millions of individuals are occupationally exposed to benzene (U.S. Department of Health and Human Services, 1983), further study of the effects of low-level benzene exposure on the immune response seems warranted.

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