

Hematotoxicity and Carcinogenicity of Inhaled Benzene

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CBA/Ca male mice have been exposed to benzene in air at 10, 25, 100, 300, 400, and 3000 ppm for variable intervals 6 hr/day, 5 days/week for up to 16 weeks. Two weeks of inhaling 10 ppm produced no hematologic effects; 25 ppm induced a significant lymphopenia. Inhalation of 100, 300, and 400 ppm produced dose-dependent decreases in blood lymphocytes, bone marrow cellularity, marrow content of spleen colony-forming units (CFU-S) and an increased fraction of CFU-S in DNA synthesis. Exposure of mice to 300 ppm for 2, 4, 8, and 16 weeks produced severe lymphopenia and decrease in marrow CFU-S. Recovery was rapid and complete after 2 and 4 weeks of exposure. After 8 and 16 weeks of exposure, recovery of lymphocytes was complete within 8 weeks. It took 16 weeks for the CFU-S to recover to that of the age-matched controls after 8 weeks of exposure and 25 weeks to recover to age-matched after 16 weeks of exposure. Inhalation of 3000 ppm for 8 days was less damaging than inhalation of 300 ppm for 80 days (same integral amount of benzene inhaled). The inhalation of 3000 ppm has not increased the incidence of leukemia or shortened its latency for development. Inhalation of 300 ppm 6 hr/day for 16 weeks significantly increases the incidence of myelogenous neoplasms in male CBA/Ca mice. Inhalation of 100 ppm for same interval does not influence incidence of myelogenous neoplasms but does increase incidence of solid neoplasms particularly in female CBA/Ca mice. Benzene is a potent carcinogen in CBA/Ca mice.

Introduction

Presumably the first reference in the literature to harmful effects from benzene is a report of a case of purpura in 1897 (1). From 1897 through 1950 there were sporadic case reports associating exposure to benzene to the development of purpura and leukemia. These are summarized by Bernard (2). These anecdotal observations clearly implicated benzene as a hematotoxin. In 1916-1917 (3,4) there were two studies on the role of benzene as a leukotoxin. These studies investigated the degeneration and regeneration of hematopoietic tissues of animals.

The first convincing studies on the possible role of benzene as a leukemogenic agent in human beings were those of Aksoy et al. (5-8) and Vigliani and Saita (9). Aksoy (5,6) reported that leukemia in benzene-exposed workers decreased after the phase-out of benzene use in Istanbul,

and there was no leukemia observed in workers during the last 3 years of the last study, implicating benzene as a probable causative agent in the earlier intervals.

Detailed epidemiological studies have been performed suggesting that there is a linear no-threshold dose-effect relationship for induction of leukemia in man (10-13), which is an unlikely relationship. There are a large number of uncertainties in the estimation of human cancer risk because many of the important conditions with which one is concerned lie outside the range of scientific observation. Infante and White (10,12,13) and the International Agency for Research on Cancer (14) adopted the unproved one-hit linear model, which assumes no threshold and that every incremental dose of benzene is accompanied by an increment in the excess cancer risk. The one-hit hypothesis assumes that there is a single, unique chemical event that is irreversible, and when this event is in DNA there is no repair of the injury. It is well known that DNA is repaired at times almost completely by known biochemical mechanisms.

Hoel et al. (15) point out that the relationship between the applied dose and incidence of cancer is frequently nonlinear, but the tumor response in some instances is linear with resultant concentration of DNA adducts in the target organ. It is reasonable to assume that benzene metabolites, at least in part, will result in DNA adducts and that it is unlikely that the incidence of leukemia will

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follow a one-hit, no-threshold response to exposure with graded concentrations of benzene. This may, however, pertain to adducts of DNA with benzene metabolites.

Previous studies on experimental induction of leukemia and other neoplasms by benzene have generally involved a lifetime exposure. The exposure regimen in the present studies was based on the apparent exposure conditions of human beings described by Aksoy et al. (5-8) and Vigliani and Saita (9). Human beings commence work in industry at 18 years or older, thus exposure in mice was commenced at 12 to 14 weeks of age, when they are sexually mature. It was assumed that human beings were exposed for 16 weeks, approximately 15% of their lives and, accordingly mice were exposed for about 15% of their lifespan. The work days were mimicked by exposing mice 6 hr/day, 5 days/week. The general objectives of these studies were a) to determine early and late effects of benzene on hematopoietic stem cells and early effects on the early hematopoietic progenitors, b) to compare the hematologic effects induced by the same total amount of benzene inhaled over short or long intervals, and c) to observe for the induction of leukemia and other neoplasms by the exposure regimen outlined above to 300 and 100 ppm of inhaled benzene.

Materials and Methods

Benzene Exposure

Mice were exposed to room air or air containing 10, 25, 100, 300, 400, or 3000 ppm benzene in chambers similar to those described by Hinners et al. (16), an isolation system described by Laskin et al. (17), or in glass jar chambers like those described by Leach (18). Benzene concentrations were monitored via an automatic gas sampling system coupled to a gas sampling loop Packard Model 417 gas chromatograph or by wet-chemical spectrophotometric analysis using a bubbler with ethanol as the absorbing solution. Mice were allowed food and water *ad libitum* except during exposure periods. Exposures were 6 hr/day, 5 consecutive days/week. When not in exposure chambers animals were housed in temperature- and humidity-controlled rooms on a 12-hr light/dark cycle. The incoming air was passed through HEPA filters and exhausted to the outside. Corn-cob bedding was sterilized before use and rooms were maintained pathogen free.

Hematopoietic Stem Cells and Early Progenitors

At various times during and after the exposure period mice were removed from the control and benzene groups, anesthetized by ether, bled from the orbital sinus, and killed by cervical dislocation. Blood counts and assays for stem cells were performed approximately 18 hr after termination of the exposure period or at the times designated. Peripheral blood values were determined with a Technicon H-1 hematology analyzer.

Hematopoietic stem cells (HSC) were assayed by

removing hind legs and flushing out the bone marrow from tibia and femur. Total marrow cellularity was determined from the resultant suspensions with a Coulter electronic counter. An aliquot of some bone marrow suspensions was subjected to tritiated thymidine (^3H Tdr) cytochrome (19) to determine fraction in DNA synthesis. The spleen colony assay of Till and McCulloch (20) was used for determination of the number of pluripotent hematopoietic stem cells in the bone marrow. Recipient mice were given 850 rad from 250 kVp, 30 mA GE Maxitron X-ray machine with 1 mm aluminum and 0.5 mm copper filtration at a dose rate of 90 to 100 rad/min for 2 hr before being used for assay. Fifty thousand nucleated bone marrow cells from the individual or pooled suspensions above were given IV. The recipients were killed 10 days after injection. The spleens were removed, fixed in Bouin's solution, and the surface colonies counted.

To test the functional capacity of the stem cells in mice exposed to benzene, a rescue assay was used. Animals were exposed for 16 weeks to 300 ppm. Benzene-exposed and sham-exposed, age-matched mice were allowed to recover for more than 2 years (752 days). At that time they were used as bone marrow donors to start a serial rescue assay in which recipients were fatally irradiated (850 rad) and then given 10^7 bone marrow cells IV from the sham- or benzene-exposed donor groups. Surviving recipients at 30 days after rescue transplant were then used as bone marrow donors for a subsequent rescue assay. This procedure was carried out for three successive rescues.

In selected experiments, assays of early progenitors of erythrocytic cells and granulocytic cells were performed. To culture the 2-day erythrocyte colony-forming units (CFU-E), the method of McLeod et al. (21) was used. Briefly, control and benzene-treated bone marrow (femurs and tibias) were harvested in minimum essential medium with 2% fetal calf serum. The suspensions were passed through 26- and 27-gauge needles successively. Total cell counts were determined by Coulter Electronic Counter. After 48 hr of incubation, the plasma clots were fixed *in situ* in 5% glutaraldehyde, washed in water, transferred to slides, and stained with benzidine and hematoxylin. Four clots per group were scored for erythrocytic colonies containing eight or more benzidine-positive cells.

Eight-day burst-forming units (BFU-E-8) were cultured in erythropoietin 2.5 units/mL and pokeweed-mitogen condition medium (2.5% solution). A pokeweed mitogen-conditioned medium was prepared as described by Johnson and Metcalf (22). The plasma clots were cultured at 37°C, 7% carbon dioxide. Clots were harvested on days 3 and 8. Half the clots were fixed in 5% glutaraldehyde and stained with benzidine and hematoxylin. Half of the remaining clots were fixed in 10% formalin and stained with acetylcholine esterase and hematoxylin in order to identify megakaryocyte-containing clots. The clots were surveyed at 7.5× magnification and a positive identification of the colonies and bursts were made at 40× magnification.

The assays for early progenitors of granulocytes and

macrophages (CFU-G, CFU-GM) were performed in agar cultures identical to the method of Pike and Robinson (23).

Mice

In studies on long-term effects of benzene on peripheral blood and HSC and leukemo-carcinogenesis, CBA/Ca BNL male and female mice were used. Exposure of mice was commenced at 84 days of age. Carcasses of dead mice were opened and fixed in 5% buffered formalin. Tissues were embedded in paraffin, sectioned at 7 μ m, and stained with hematoxylin and eosin. In the serial rescue and some other HSC studies, C57BL/6BNL male and female mice were used. For some studies on the effects of benzene of hematopoietic stem cells and early hematopoietic progenitors, Hale-Stoner BNL mice were used.

Results

Hematopoietic Stem Cells

These observations have been published previously (24,25). After exposure to 400 ppm benzene, 6 hr/day, the nucleated bone marrow cell count and the CFU-S per leg decreased to about 25% of the age-matched controls after five exposure intervals. Concomitantly, the fraction of CFU-S in DNA synthesis increased after 5 days of exposure to 40 to 60% compared to 20% of the age-matched controls. This was maintained through 9½ weeks of exposure. After termination the fraction in DNA synthesis diminished to the age-matched control level by 15 days. There was no significant increase during this interval in the CFU-S level.

In other studies (25), C57BL/6 mice were exposed to 10, 25, 100, 300, or 400 ppm benzene, 6 hr/day, 5 consecutive days/week, for 2 to 16 weeks. These studies showed no effect on marrow cellularity after 10 or 25 ppm, with a significant diminution in marrow cellularity at exposure to 100 ppm for 2 weeks. Similarly, there was a dose-dependent effect on diminution of the CFU-S after 100 and 400 ppm. There was also a significant increase in the fraction in DNA synthesis after 10 exposures to 100 and 400 ppm. Two weeks (10 exposures) to 10, 25, 100, and 400 ppm produced a dose-dependent diminution in lymphocytes but no effect upon granulocytes in the peripheral blood. Two weeks after exposure for 2 or 4 weeks, the stem cells had returned to the normal age-matched control level. After 8 weeks of exposure, it took 16 weeks for CFU-S to return to that of the age-matched controls. After 16 weeks of exposure there was a slow, nearly linear return for 8 weeks with no further increase at 16 weeks after exposure, but by 25 weeks the level was that of the age-matched controls. The degree of lymphopenia was nearly the same after 2, 4, 8, and 16 weeks of exposure with rapid recovery by 8 weeks after exposure to that of the age-matched control animals.

Early Erythrocytic Progenitors

Figure 1 shows the effect of exposure to 400 ppm, 6

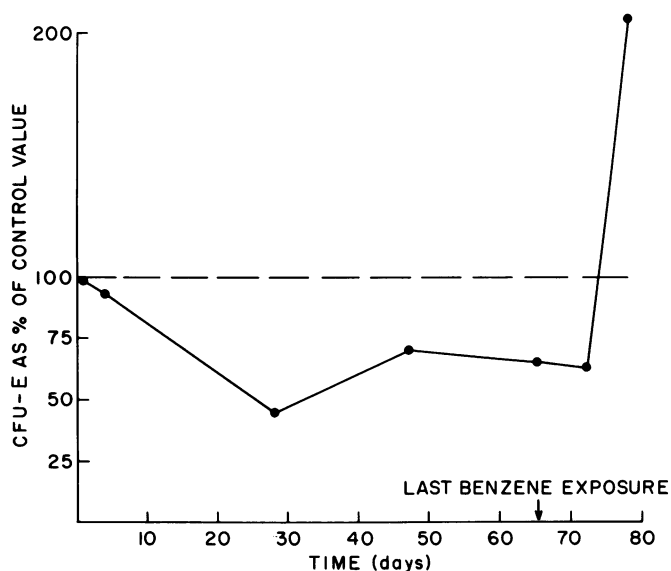


FIGURE 1. Serial changes in the number of plasma clot CFU-E in pooled bone marrow of Hale-Stoner mice inhaling 400 ppm benzene 6 hr/day, 5 days/week for 47 exposures (9½ weeks).

hr/day, 5 days/week, in Hale-Stoner mice. After one and four exposures there was no significant change in the CFU-E. However, after 29 days, 48 days, and 65 days there was a significant diminution in the number of CFU-E. Seven days after termination of exposure there had been no recovery, but by 12 days after exposure there had been a marked increase to greater than twice that of the age-matched controls.

Figure 2 illustrates the changes seen in the BFU-E 8-day plus the erythrocytic megakaryocyte-containing

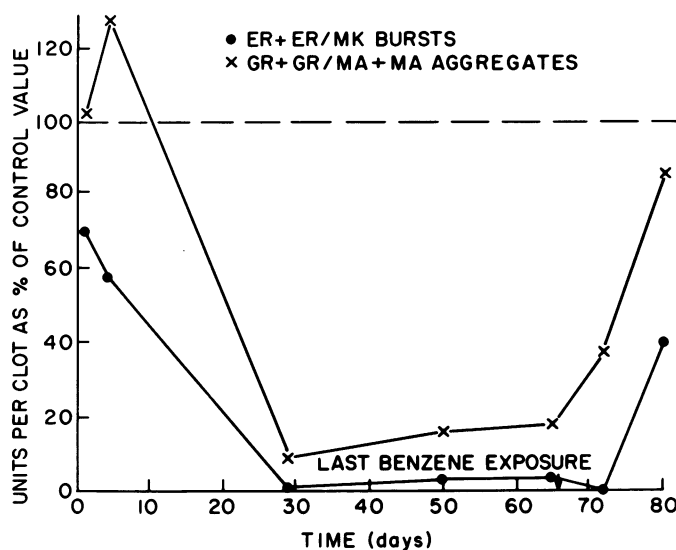


FIGURE 2. Serial changes in 8-day plasma clot cultures of 8-day BFU-E (o) and granulocyte macrophage aggregates (x) in pooled bone marrow of Hale-Stoner mice inhaling 400 ppm benzene 6 hr/day, 5 days/week for 47 exposures (9½ weeks). ER + ER/MK bursts, erythroid and erythroid megakaryocytic bursts; GR + GR/MA + MA aggregates, granulocytic and granulocytic/macrophages and macrophage aggregates.

bursts. The first culture was 90% of the age-matched control after one exposure. By 29 days after commencement of exposure (22 exposures) very few 8-day bursts remained assayable. Recovery was incomplete 12 days after termination of exposure.

Figure 2 also illustrates the changes in granulocyte-macrophage aggregates seen in the plasma clots. There is a smaller decrease in their number with recovery to nearly that of the age-matched controls 12 days after termination of exposure.

Figure 3 illustrates the effect upon the CFU-GM in agar culture plates. The data are presented as percent of control and actual number of colonies counted in the plates from the benzene-treated and the age-matched control mice. There is a suggestion of an increase in the CFU-GM after commencement of exposure, but by 30 days there is a significant diminution and an apparent partial recovery during continued exposure.

The rather marked changes in the assayable, early erythropoietic progenitors was reflected in a decrease in the red blood cell counts during the exposure to about 50 to 75% of the age-matched controls. There were lesser, oscillating changes in the granulocyte level in the blood.

Figure 4 shows the results from the three successive serial rescue transplants. The effectiveness of bone marrow from sham-exposed donors was compared to that of bone marrow from mice that had been exposed to benzene 752 days earlier. All transplants consisted of 10^7 bone marrow cells. In the first rescue of mice that had received 850 rad, the 30-day survival was 100%. At 290 days after rescue, 7% of the mice receiving bone marrow from sham-exposed donors had died and 36% had died of those receiving bone marrow from benzene-exposed donors. Again, in the secondary transplant there were no 30-day deaths in the mice receiving bone marrow from

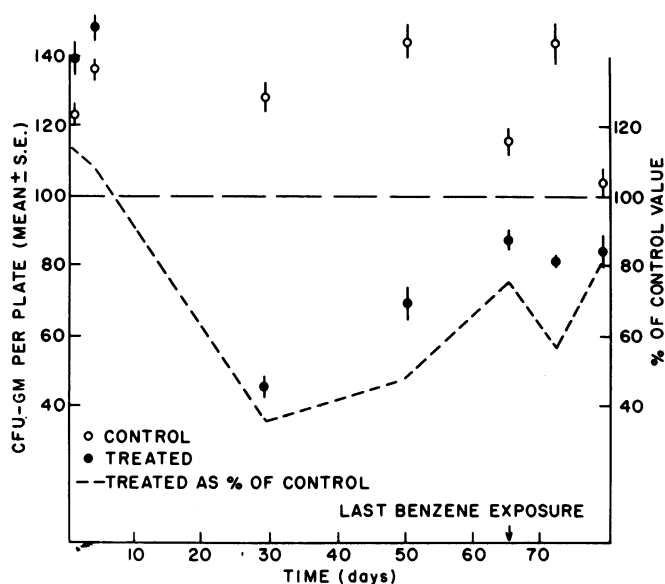


FIGURE 3. Serial changes in 7-day agar culture of GM-CFU-C in bone marrow of Hale-Stoner mice inhaling 400 ppm benzene 6 hr/day, 5 days/week for 47 exposures (9½ weeks).

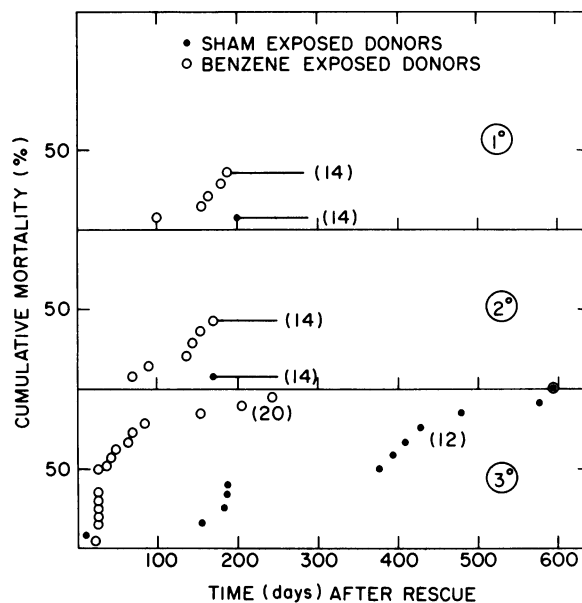


FIGURE 4. Serial rescue bone marrow transplantation from donor C57BL/6BNL mice that had been exposed to benzene 300 ppm 6 hr/day, 5 days/week for 16 weeks 752 days earlier. 1°, 2°, 3°, primary, secondary, and tertiary rescues in the series. Numbers in parentheses are numbers of animals set aside for long-term survival.

sham-exposed or benzene-exposed mice. However, deaths commenced at 70 days after rescue in those animals receiving bone marrow from the benzene-exposed donors whereas there were no deaths in the mice receiving bone marrow from the sham-exposed until about 171 days after exposure. In the tertiary rescue the recipients of the marrow from the original benzene-exposed donors commenced dying early, and 50% were dead by 30 days after exposure. By 245 days after exposure 95% were dead, and the last animal died at 595 days after radiation. In contrast, with the mice receiving marrow from the original sham-exposed, there was only one death during the first 30 days with 50% of the animals surviving about 375 days and the last animal dying at 594 days after irradiation.

Hematologic Effects of the Same Amount of Benzene Inhaled over Short or Long Periods

It was originally intended to compare the effect of inhalation of benzene over 2 days compared to inhalation of the same total amount over 20 days. To allow assays 1 day following final exposure, 316 ppm, 6 hr/day, 5 days/week for a total of 19 exposures, was compared to 3000 ppm, 6 hr/day for two successive exposures. Figure 5 shows the effects upon absolute neutrophil and lymphocyte counts in the blood. There is a drastic reduction in the lymphocyte count after 19 exposures to 316 ppm from 7500 lymphocytes to 300 lymphocytes per microliter. Two-day exposure to 3000 ppm resulted in a reduction from 6700 in the sham-exposed to 3300/ μ L in the benzene-exposed 1 day after the end of exposure.

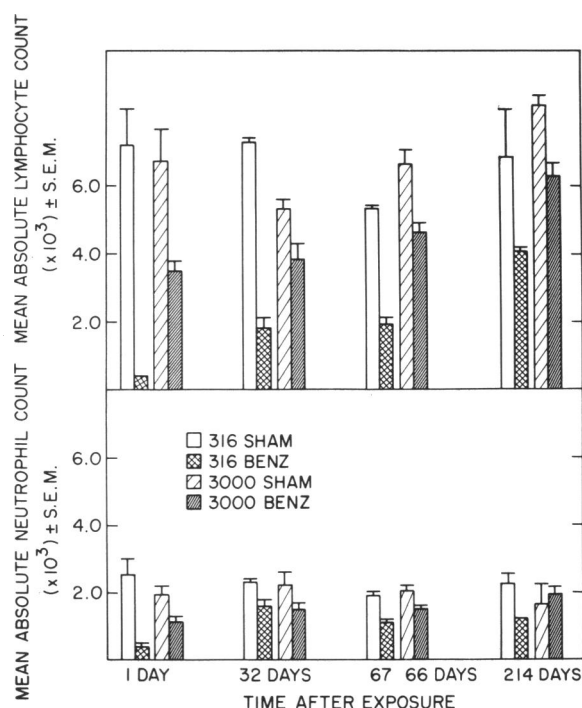


FIGURE 5. Comparison of blood neutrophil and lymphocyte counts in CBA/Ca BNL male mice that had inhaled the same total amount of benzene over 19 or 2 days.

Thirty-two days after exposure there was still a marked reduction in the lymphocyte level in the animals receiving 316 ppm over 19 days compared to the mice receiving 3000 ppm in two exposures. These differences still persisted at 66 to 67 days and 214 days after exposure (Table 1).

The neutrophil counts 1 day after exposure showed a significantly more severe neutropenia in the mice receiving 316 ppm for 19 exposures than in the mice receiving 3000 ppm in two exposures. Thirty-two days after exposure and 66 to 67 days after exposure a slight neutropenia was still present with no significant difference between the two exposure regimens. By 214 days after exposure the mice that had been exposed to 3000 ppm had recovered, but those that had been exposed to 316 ppm were still neutropenic.

Figure 6 shows the results of inhaled benzene on marrow cellularity and CFU-S content. One day after termi-

nation of exposure, the reduction in cellularity in the animals receiving 316 ppm for 19 days was significantly greater than that of the mice that received 3000 ppm over 2 consecutive days. At 32, 66 to 67, and 214 days after exposure cellularity had returned to that of the sham-exposed mice. The reduction in CFU-S content 1 day after exposure in the mice receiving 316 ppm for 19 days was substantially and significantly greater than in the animals receiving 3000 ppm for 2 consecutive days. Thirty-two days after exposure the CFU-S content of the marrow in the mice receiving 316 ppm was significantly less than that of the sham-exposed animals, whereas the mice that had received 3000 ppm for 2 consecutive days the CFU-S had returned to that of the sham-exposed animals. In the 300-ppm group CFU-S content of marrow returned to sham levels by 66 days after exposure. However, in the 316-ppm group the CFU-S remained significantly below the sham levels at 214 days after exposure.

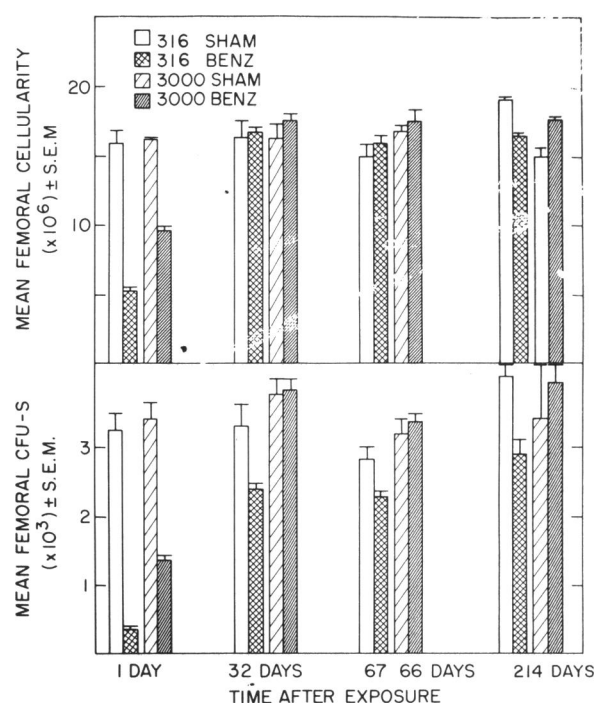


FIGURE 6. Comparison of bone marrow cellularity and femoral CFU-S in CBA/Ca BNL male mice that received the same total amount of benzene over 2 days (3000 ppm) or 19 days (316 ppm).

Table 1. Comparison of differential leukocyte counts in sham-exposed CBA/Ca BNL mice and mice that inhaled 316 ppm for nineteen 6-hr exposures.^a

Time after treatment	Group	WBC, $\times 10^3/\mu\text{L}$	Neutrophil, $\times 10^3/\mu\text{L}$	Lymphocyte, $\times 10^3/\mu\text{L}$	Monocyte, $\times 10^3/\mu\text{L}$	Eosinophil, $\times 10^2/\mu\text{L}$	LUC, $\times 10^2/\mu\text{L}^b$
1d	Sham	10.4 $\pm$ 0.9	2.2 $\pm$ < 0.1	7.5 $\pm$ 1.0	4.0 $\pm$ 0.6	2.6 $\pm$ 0.5	0.4 $\pm$ 0.1
	Benzene	0.8 $\pm$ < 0.1	0.3 $\pm$ < 0.1	7.1 $\pm$ 0.3	2.5 $\pm$ 0.5	0.1 $\pm$ 0.1	0.2 $\pm$ < 0.1
32d	Sham	10.3 $\pm$ 0.9	2.0 $\pm$ 0.2	7.6 $\pm$ 0.7	3.9 $\pm$ 0.5	2.4 $\pm$ 0.4	0.4 $\pm$ 0.1
	Benzene	3.4 $\pm$ 0.4	1.4 $\pm$ 0.2	1.5 $\pm$ 0.2	2.7 $\pm$ 0.3	1.9 $\pm$ 0.5	1.0 $\pm$ 0.5
67d	Sham	7.9 $\pm$ 0.1	1.9 $\pm$ 0.1	5.3 $\pm$ 0.2	3.3 $\pm$ 0.2	2.8 $\pm$ 0.6	0.5 $\pm$ 0.2
	Benzene	3.3 $\pm$ 0.3	1.1 $\pm$ 0.1	1.9 $\pm$ 0.2	1.7 $\pm$ 0.3	1.1 $\pm$ 0.1	0.3 $\pm$ 0.2
214d	Sham	10.0 $\pm$ 0.2	1.8 $\pm$ 0.1	7.1 $\pm$ 1.3	8.0 $\pm$ 1.8	2.2 $\pm$ 0.6	0.8 $\pm$ 0.3
	Benzene	5.5 $\pm$ 1.1	0.9 $\pm$ 0.1	3.9 $\pm$ < 0.1	5.9 $\pm$ < 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0.5

^aValues are means $\pm$ SE.

^bLUC, large, unstained cell, mainly large lymphocytes.

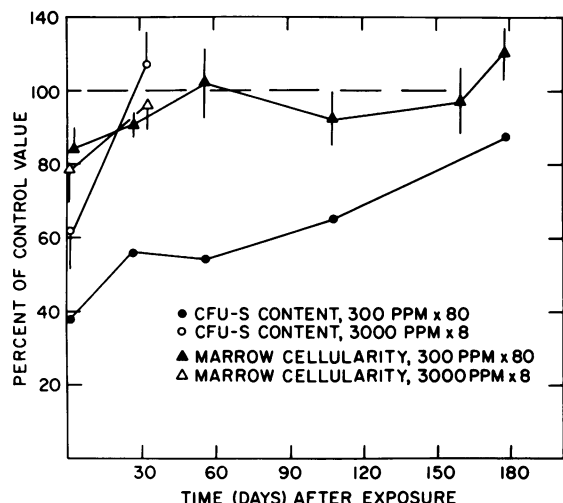


FIGURE 7. Femoral cellularity and CFU-S content as percent of the sham-exposed mice after inhalation of 3000 ppm benzene for eight 6-hr sessions or 300 ppm for eighty 6-hr sessions (C57BL/6BNL females).

Table 1 shows the actual differential leukocyte counts in number per microliter in CBA/Ca BNL mice that inhaled 316 ppm benzene for nineteen 6-hr sessions. All of the cell types are reduced in number as compared to the sham-exposed mice at 1 day. All absolute counts except the large unstained cells (LUC) are depressed at 32, 67, and 214 days after exposure. Figure 7 shows the serial changes in the bone marrow cellularity and CFS-S in C57B1/6BNL mice that inhaled 300 ppm for eighty 6-hr sessions compared to mice that inhaled 3000 ppm for eight sessions—the same total amount over one-tenth the time.

The marrow cellularity and CFU-S of mice that inhaled 3000 ppm for eight sessions are depressed one day after termination of exposure but rebound to age-matched control by 30 days after exposure. The decrease in marrow cellularity of mice that inhaled 300 ppm for eighty sessions is nearly the same as in mice that inhaled 3000 ppm for eight sessions, but it takes 60 days to return to the level of the age-matched, sham-exposed controls. The CFU-S is decreased to 38% of sham-exposed controls in mice that inhaled 300 ppm compared to 62% in the mice that inhaled 3000 ppm. The recovery in the CFU-S was much slower and incomplete 178 days after exposure.

Mortality and Neoplasia

Figure 8 shows the mortality in sham- and benzene-exposed male and female CBA/Ca BNL mice inhaling 300 ppm benzene 6 hr/day, 5 days/week for 16 weeks. Deaths commenced in the sham-exposed males at about 600 days of age with a median lifespan of 1030 days compared to deaths commencing during exposure in the male mice with a median lifespan of 510 days. The sham-exposed females commenced dying at about 680 days of age with a median life-span of 1100 days. This is compared to the female exposed mice that commenced dying shortly after end of exposure with a median lifespan of 580 days. Unfortunately, the carcasses of the male mice that died dur-

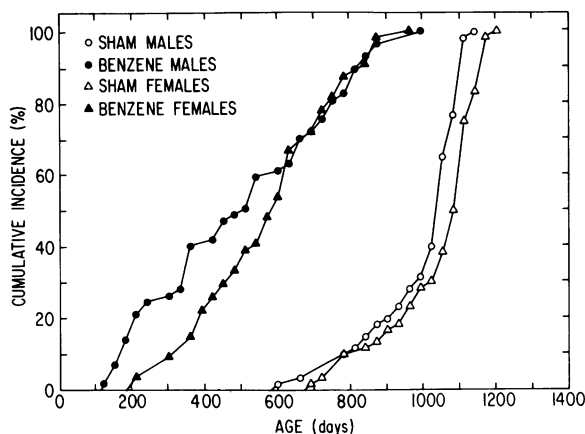


FIGURE 8. Mortality curves for male and female CBA/Ca BNL mice that inhaled 300 ppm benzene 6 hr/day, 5 days/week for 16 weeks compared to the sham-exposed mice.

ing exposure were not saved. It is assumed without proof that they were dying from the sequelae of marrow hypoplasia.

Figure 9 shows the appearance of myelogenous neoplasms, which is the sum of neoplasms resembling acute myeloblastic and chronic granulocytic leukemia. None were observed in the sham-exposed males. The benzene-exposed males began to develop myelogenous neoplasms shortly after exposure, attaining a 19.3% lifetime incidence at about 530 days of age. Females developed myelogenous neoplasms later that first appeared at about 350 days of age with a lifetime incidence of 11.1%, compared to a lifetime incidence in the sham-exposed females of 2%. See Table 2 for statistical significance of differences.

Figure 10 shows the appearance of lymphomatous neoplasms, which is the sum of solitary lymphomas, diffuse nonthymic non-Hodgkins lymphoma, and Hodgkins lymphomata. This is a late-appearing spectrum of diseases in

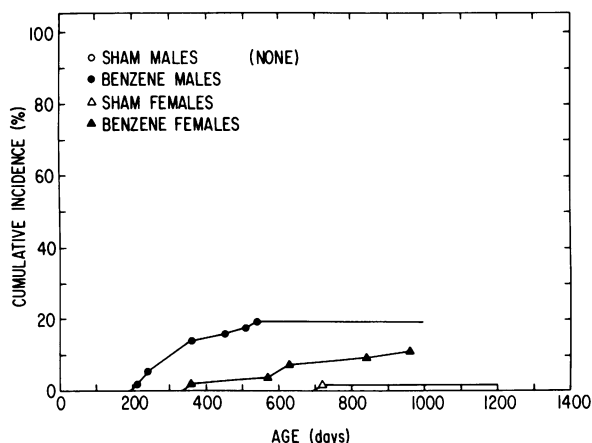


FIGURE 9. Incidence curves for myelogenous neoplasms (acute myeloblastic and chronic granulocytic leukemia) for male and female CBA/Ca BNL mice after inhalation of 300 ppm benzene for eighty 6-hr sessions.

Table 2. Final incidence (%) of various neoplastic conditions among CBA/Ca BNL mice that inhaled 300 ppm benzene or were sham-exposed.

Condition	Sham males, <i>n</i> = 60	Benzene males, <i>n</i> = 57	Sham females, <i>n</i> = 60	Benzene females, <i>n</i> = 54
Lymphomatous neoplasms	11.7	1.8 (0.037) ^a	8.3	7.4 (> 0.200)
Myelogenous neoplasms	0.0	19.3 (< 0.001)	1.7	11.1 (0.040)
Hepatomata	26.7	10.5 (0.028)	13.3	0.0 (0.006)
Neoplasms other than hepatomata and hematopoietic	21.7	52.6 (< 0.001)	35.0	79.6 (< 0.001)

^aNumbers in parentheses are *p* values, χ^2 test.

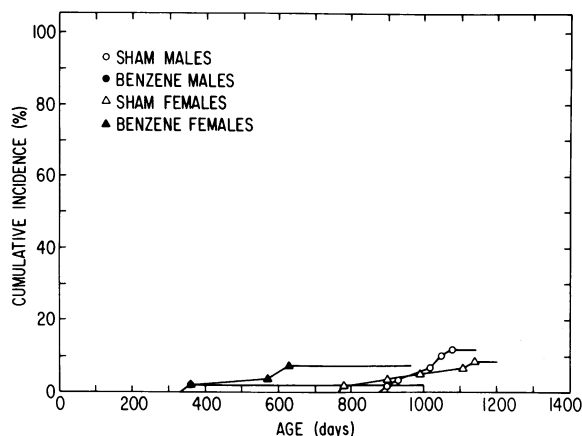


FIGURE 10. Incidence curves for lymphomatous neoplasms (Hodgkins, non-Hodgkins lymphoma, and reticulum cell sarcoma) for CBA/Ca BNL male and female mice that inhaled 300 ppm benzene 6 hr/day, 5 days/week for 16 weeks compared to the sham-exposed mice.

the sham-exposed animals with first appearance at about 880 days of age in the males attaining a lifetime incidence of 11.7% in the males and 8.3% in the females. In both sexes benzene exposure results in an earlier onset in the appearance of lymphomatous neoplasms to a lifetime incidence of 7.4% in the females and 1.8% in the males. The lifetime incidence is significantly lower in the benzene-exposed males, and there is no significant difference in the benzene-exposed females when compared with their respective sham-exposed cohorts. It should be noted that lymphomatous neoplasms occur spontaneously late in the mouse's life. Due to the high mortality in the benzene-exposed mice, few animals lived to an age at which these diseases normally appear. See Table 1 for statistical significance.

Figure 11 shows the occurrence of nonhematopoietic and nonhepatic neoplasms of male and female mice that inhaled 300 ppm of benzene for 6 hr/day, 5 days/week, for 16 weeks. These neoplasms are primarily a combination of Harderian and Zymbal gland, squamous cell carcinoma, mammary adenocarcinoma, and papillary adenocarcinoma of the lung. In sham-exposed male mice these tumors commenced at 780 days of age with a lifetime incidence of 21.7%. In the benzene-exposed males appearance commenced shortly after cessation of exposure reaching a lifetime incidence of 52.6% ($p > 0.001$). In the female sham-exposed (incidence 35.0%) these tumors commenced appearance at about 900 days of age. In the

benzene-exposed females these tumors commenced appearance at 360 days of age with a lifetime incidence of 79.6% ($p > 0.001$).

Among males exposed to benzene that died after completion of exposure and which were neoplasm-free on autopsy, one-half (3/6) were found to have hypoplastic or aplastic bone marrow. This condition was first found at 339 days of age, 143 days after completion of exposure. Among sham-exposed males, however, while the incidence was similar (13/28), the first appearance was not until 580 days of age.

Among benzene-exposed females (1/4), the first incidence of marrow failure came at 480 days of age while among sham-exposed females, though the incidence was similar (2/7), the age at onset was much later (949 days of age). These findings show that in animals that died without neoplasm bone marrow hypoplasia/aplasia occurred much earlier in benzene-treated groups.

Figure 12 shows the comparison of mortality in male CBA/Ca BNL mice that inhaled 100 ppm 6 hr/day, 5 days/week, for 16 weeks. The sham-exposed mice commenced to die at 630 days of age compared to deaths during exposure to benzene. Unfortunately, the carcasses were not saved, so cause of death during exposure is not known. The median survival is 1020 days in sham-exposed compared to 1000 days in benzene exposed. The

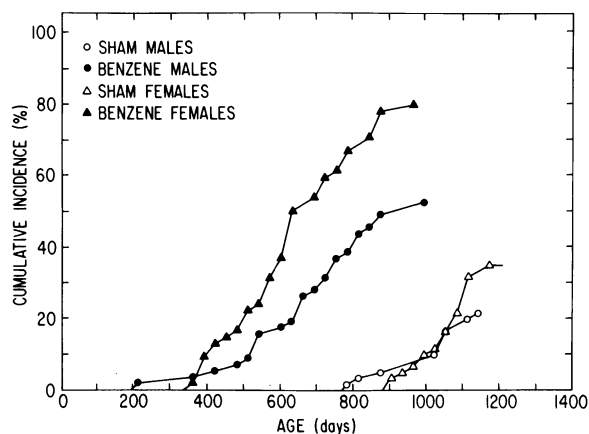


FIGURE 11. Incidence curves for neoplasms other than hematopoietic and hepatic (Zymbal, Harderian, mammary adenocarcinoma breast, squamous cell carcinoma, papillary adenocarcinoma of lung, and benign tumors) for male and female CBA/Ca BNL mice that inhaled 300 ppm benzene 6 hr/day, 5 days/week for 16 weeks compared to the sham-exposed mice.

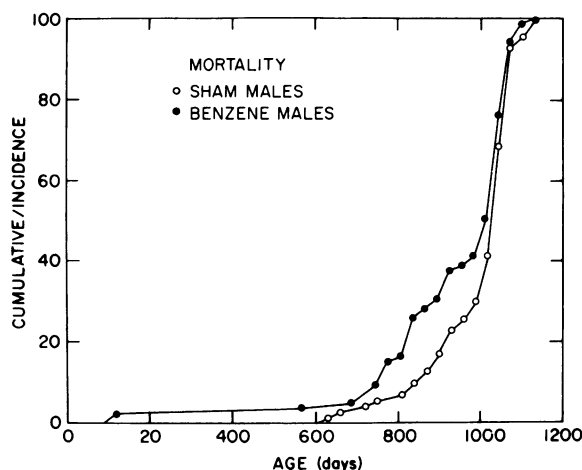


FIGURE 12. Incidence curves for mortality of male CBA/Ca BNL mice that inhaled 100 ppm benzene 6 hr/day, 5 days/week for 16 weeks compared to sham-exposed mice.

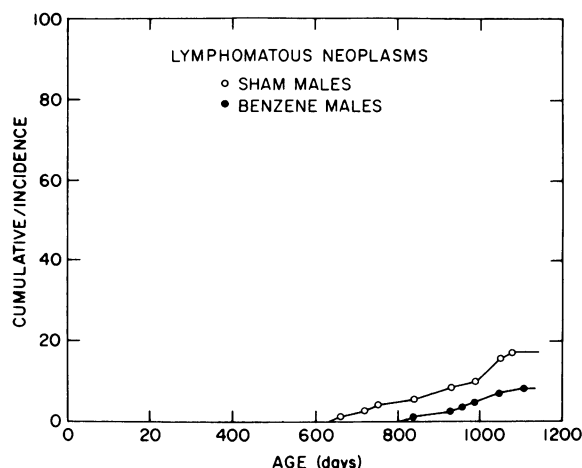


FIGURE 13. Incidence curves for lymphomatous neoplasms in CBA/Ca BNL male mice that inhaled 100 ppm benzene 6 hr/day, 5 days/week for 16 weeks compared to the sham-exposed mice.

25% mortality in sham-exposed was attained at 960 days, compared to 840 days in the benzene exposed.

Figure 13 compares the incidence of lymphomatous neoplasms in sham- and benzene-exposed male mice (100 ppm). The benzene-exposed mice had a lifetime incidence of 8.2% compared to 17.1% in the sham-exposed mice, indicating that benzene exposure decreased the incidence of lymphomata ($p = 0.1$).

Figure 14 compares the incidence curves for non-hematopoietic nonhepatic neoplasms in male CBA/Ca BNL mice that had inhaled 100 ppm benzene. The lifetime incidence in sham-exposed was 20% compared to 44.7% in mice that inhaled benzene (100 ppm) ($p > 0.001$).

Incidence curves for hepatomata in male and female mice show a lower incidence in male mice inhaling 300 ppm 26.7% compared to 10.5% ($p < 0.028$). In females the difference was more striking 13.3% (sham) to 0% in 300 ppm ($p = 0.006$). There was no significant difference in male mice inhaling 100 ppm for 16 weeks.

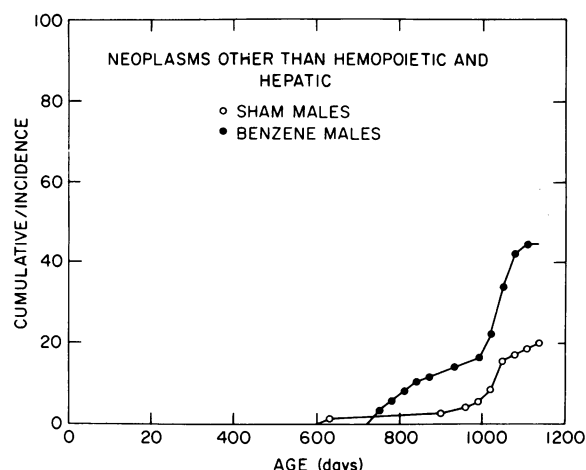


FIGURE 14. Incidence curves for neoplasms other than hematopoietic and hepatic (Zymbal, Harderian, mammary adenocarcinoma breast, squamous cell carcinoma, papillary adenocarcinoma of lung, and benign tumors) for male and female CBA/Ca BNL mice that inhaled 100 ppm benzene 6 hr/day, 5 days/week for 16 weeks compared to the sham-exposed mice.

Table 2 lists the final incidence of the different groups of neoplasms and the probability of a significant difference from the incidence in sham-exposed male and female CBA/Ca mice. In both sexes benzene (300 ppm) induced a significant increase in myelogenous neoplasms and non-hematopoietic nonhepatic neoplasms. In contrast to this the final incidence of hepatomata is significantly decreased by inhalation of benzene (300 ppm) in both sexes and of lymphomata in males but not females.

Table 3 lists the final incidence of neoplasms in CBA/Ca male mice that inhaled 100 ppm benzene for 16 weeks. There is a lesser incidence of lymphomata in the benzene exposed mice of borderline significance. There is no significant difference in myelogenous or hepatic neoplasms. Neoplasms other than hemopoietic and hepatic neoplasms are significantly increased ($p = 0.001$).

Last, in studies still in progress using both female C57B1/6 BNL and male CBA/Ca BNL mice, inhaling 3000 ppm for 8 days has not been leukemogenic or carcinogenic. Although it would be premature to draw any final conclusions, clearly, the early and high incidences of mortality and neoplasms seen in comparable 300 ppm-for-80 days experiments have not occurred. These animals are now approaching 700 days of age.

Table 3. Final incidence (%) of various neoplastic conditions among CBA/Ca BNL mice exposed to 100 ppm benzene- or sham-exposed.

Condition	Sham males, $n = 70$	Benzene males, $n = 85$
Lymphomatous neoplasms	17.1	8.2 (0.10) ^a
Myelogenous neoplasms	0.0	2.4 (0.19)
Hepatomata	38.6	41.2 (< 0.20)
Neoplasms other than hepatomata and hematopoietic	20.0	44.7 (0.001)

^aNumbers in parentheses are p values, χ^2 test.

Discussion

There have been many reports on the effect of benzene on hematopoietic stem cells and early progenitors (24–31). All studies show that benzene inhalation of 100 ppm or more reduces the number of hematopoietic stem cells and early progenitors. Baarson et al. (28) showed that exposure to 10 ppm 6 hr/day for 5 days/week for 178 days reduces the BFU-E to 55% of controls after 66 days with a return to normal despite continued exposure. CFU-E fell to 5% of the level in sham-exposed mice with only a modest decrease in red blood cell levels in the blood. If the CFU-E is an obligatory pathway to the circulating red cell, one would have expected a severe anemia 15% of normal. As this did not happen, one wonders if the benzene metabolites interfere with synthesis or function of the CFU-E receptors so that the *in vitro* stimulating molecules are ineffective. If the *in vitro* assays are correct, then the few remaining CFU-E must have a much greater amplification or the *in vitro* CFU-E may not represent an obligatory step *in vivo*. Baarson et al. (unpublished) found that consumption of 5% ethanol in drinking water enhanced the effect of 10 ppm benzene in air. Seidel et al. (31) exposed mice to 300 ppm 6 hr/day, 5 days/week, for 16 weeks and observed changes in the CFU-E and CFU-E of lesser magnitude than Baarson et al. (28). Whether these represent strain differences or differences in sensitivity of the *in vitro* cultures is unclear. Seidel et al. (31) also showed that after 4 weeks of sham exposure and benzene (300 ppm) exposure mice had almost an equal reticulocyte response to bleeding, suggesting that anemia to a large extent may be due to hemolysis. We have observed intense hemosiderosis in spleens indicating hemolysis. Our studies in Hale-Stoner male mice exposed to 400 ppm benzene for 9.5 weeks did not develop as severe a decrease in CFU-E as in the Baarson et al. (28) study. Whether this is because of strain differences or to differences in assay system is not known.

Moeschlin and Speck (29) and Kissling and Speck (30) concluded that administration of benzene results in inhibition of DNA synthesis in the bone marrow. The overall reduction of DNA synthesis in the bone marrow may be the result of cell killing. Cronkite et al. (24,25) focused on the hematopoietic stem cells determining the fraction in DNA synthesis by ³H-thymidine cytocide. There is a marked increase in the fraction of CFU-S in DNA synthesis compensating for the reduced number of CFU-S in the bone marrow, not a decrease in fraction in DNA synthesis as reported earlier.

The serial transfer of syngeneic bone marrow in irradiated hosts was first reported by van Bekkum and Weyzen (32). Mice are fatally irradiated and then rescued by large bone marrow transplant of 10⁶ to 10⁷ syngeneic bone marrow cells. At variable times after irradiation and rescue bone marrow transplantation, the survivors are used for donors in the second rescue. This is repeated until the bone marrow that has never been irradiated becomes exhausted. This has been termed decline in colony-forming ability of marrow cells subjected to serial transplantation into irradiated mice by Siminovitch, Till, and

McCulloch (33). This phenomenon of decline has been studied by numerous investigators (33–41). Most investigators believe that the decline is due to exhaustion of the finite mitotic capacity of the HSC. Our studies (unpublished) indicate that prolonged rests of 100 to 200 days after three rescue transplants allow the HSC to regain their mitotic capacity. Brecher et al. (unpublished) believe decline in mitotic capacity is due to malfunction of the marrow stroma in failing to provide adequate hematopoietic molecular regulators. Studies reported herein clearly show that bone marrow from mice that have previously been exposed to benzene for 752 days is less effective in maintaining long-term survival of rescued mice and declines more rapidly than the bone marrow from the sham-exposed donors. Alternative explanations are possible. The decline may be due to a) a long-standing genetic injury to the pluripotent hematopoietic stem cells by failure to repair DNA adducts or other injury to DNA; b) exhaustion of the G₀ stem cells by increasing the fraction that were in DNA synthesis during the 16 weeks of exposure to benzene thus exhausting the ultimate mitotic capacity of the G₀ hematopoietic stem cells by repetitive mitoses early in life; and c) a radiation effect on the stromal cells of the recipient as suggested by Brecher (unpublished). This would suggest that the benzene-treated stem cells are more susceptible to stromal defect.

The inhalation of 316 ppm benzene for 19 days from an exposure standpoint is equivalent to inhaling 3000 ppm for 2 days (each equivalent to 6000 ppm days). However, there are large differences in the hematologic effects. The longer exposure to a lower concentration of benzene results in a much greater hematotoxic effect. The greater effect of the lower concentration of benzene over a longer interval might be due to saturation of the enzymes that metabolize benzene when inhaling 3000 ppm whereas inhaling 300 ppm rather than 3000 ppm allows a larger fraction of the benzene to be metabolized. This question might be answered by measuring total phenol production in both instances. A comparison of the effects of single doses of ionizing radiation with chronic exposure to ionizing radiation is of collateral interest. Single doses of ionizing radiation are much more effective in cell killing and carcinogenesis than the same amount of ionizing radiation protracted over a longer time. This is generally assumed to be due to repair of injury to DNA.

The reverse may be the case with exposure to benzene with irreparable injury building up with time. In the case of 3000 ppm benzene exposure for 6 hr/day, there is a daily 18-hr interval for repair. In the case of nineteen 6-hr exposures to 300 ppm benzene 5 days/week, there is also a daily 18-hr interval for repair and 60 hr from 4:00 P.M. on Friday to 8:00 A.M. Monday. In the former case there is either lesser injury or more repair. It had been intended to repeat studies at more concentrations to see if prolongation of exposure to the same total amount of benzene would continue to have a greater hematotoxic effect and whether it would have a greater leukemogenic effect.

The inhalation of 300 ppm 6 hr/day, 5 days/week for 16 weeks drastically reduces the median survival time and maximum survival time. A large fraction of the reduction

in survival time is due to an earlier onset and higher incidence of malignant neoplasms. The deaths that occurred during exposure are presumed due to the sequelae of bone marrow hypoplasia, but this is not known since the carcasses were not saved. This assumption is supported by the observation of earlier marrow hypoplasia in benzene-exposed mice. In males and females there is a highly significant increased incidence of myelogenous neoplasms occurring earlier in life. There is also a very significant, earlier and higher incidence of neoplasms other than hematopoietic and hepatic. These neoplasms constitute a broad spectrum of tumors including squamous cell carcinoma, mammary adenocarcinoma, Zymbal and Harderian gland tumors, and papillary adenocarcinoma of the lung. Lymphomatous neoplasms appear earlier in the mice exposed to benzene than in the sham-exposed mice. Lifetime incidence is slightly less in the case of the benzene-exposed females, but in the case of the males is significantly less.

Hepatic neoplasms appeared sooner in the benzene-exposed mice with strikingly reduced lifetime incidence. The reduction in incidence of specific types of neoplasms in the mouse by benzene exposure is apparently a new observation. The phenomenon, however, was first observed by Hellman et al. (42) in the Fischer rat exposed to ionizing radiation. This rat has a high spontaneous incidence of acute myelogenous leukemia. Exposure to ionizing radiation drastically reduces the incidence of myelogenous leukemia, but increases the incidence of mammary adenocarcinoma. They termed this a paradoxical response to ionizing radiation. Presumably, this is due to radiosensitivity of the target cells for leukemia that would produce the neoplasms spontaneously. It is assumed that a comparable mechanism is operating in the reduction by benzene of the incidence of neoplasms in CBA/Ca BNL mice that have a high spontaneous incidence. Stoner, Drew, and Bernstein (43) conducted lifetime benzene exposures to hairless mice. The incidence of leukemia was not increased. In fact, an interpretation of their data suggests that exposure to benzene by inhalation may have suppressed the development of leukemia.

The first experimental demonstration of carcinogenesis by benzene is that of Maltoni and Scarnato (44), who administered large amounts of benzene by stomach tube for long periods of time in the Sprague-Dawley rats. They developed a wide spectrum of malignant and nonmalignant neoplasms.

Snyder et al. (45) exposed C57BL/6 and AKR mice to 300 ppm, 6 hr/day, 5 days/week for a lifetime. There was a significant increase in the incidence of leukemia and lymphoma. In studies on rats exposed to 100 ppm for lifetime, a single case of chronic myelogenous leukemia, a rarity in the rat, was observed by Goldstein et al. (46). Another study by Snyder et al. (47) showed severe hematotoxicity after inhaling 100 ppm benzene in air, but no significant increase in hematopoietic neoplasms. There was a significant increase in the nonhematopoietic neoplasms. A recent monograph on benzene carcinogenicity by Askoy (48) repeatedly points out that there is no animal model for benzene induction of acute myelogenous

leukemia. Our studies correct this deficiency.

Harigaya et al. (49) adopted another approach for the detection of hematotoxicity of benzene and possible carcinogenic effect. In these studies C57BL/6J mice were exposed to 400 ppm benzene 6 hr/day, for either 9 days or for 11 consecutive days. Long-term bone marrow cultures of bone marrow from benzene-exposed mice showed a progressive reduction in the number of spleen-colony forming cells compared to bone marrow from control mice. Different combinations of adherent cells and reinoculated cells showed that cultures containing bone marrow cells from benzene-exposed mice had a lower capacity to maintain stem cell proliferation in normal combinations irrespective of whether benzene-exposed marrow was in the adherent cell layers or reinoculated cells. Clearly, benzene inhalation of this magnitude produces stem cell injury leading to diminished self-replication and derangement of the adherent marrow population. The long-term marrow cultures from the benzene-treated mice did not develop detectable transformation *in vitro* throughout the culture period. Neonatal mice inoculated with cultured cells from the benzene-exposed mice did not develop leukemia during an 8-month period after inoculation when the study was terminated. Subsequent studies suggest that a longer exposure of donor mice and a longer observation of the recipient mice may be indicated.

It has been known for years by the present investigators that four strains of mice developed an increased incidence of acute myelogenous leukemia after exposure to ionizing radiation (50-54). The RFM strain is less desirable because of expense and it has a high spontaneous incidence of acute myeloblastic and other leukemias. The C3H/He male mouse has a low spontaneous incidence of acute myeloblastic leukemia, but the female has such a high and early incidence of mammary neoplasms that it would be undesirable. The SJL/J mouse is rather expensive and less easily obtained in numbers. The CBA/H male mouse in England has a very low incidence of acute myeloblastic leukemia (< 1%), but responds to exposure to ionizing radiation with a high incidence. The CBA/Ca from Jackson Laboratories comes from the same stock as the CBA/H in England and was therefore chosen as the best and most economical of the four strains known to develop acute myeloblastic leukemia. The choice appears to have provided a good animal model for the study of benzene leukemogenesis and should be suitable for establishment of the needed benzene dose-effect curves for acute myelogenous and other leukemias. Our data suggest that there may be an effective time-dose relation (or concentration) of benzene that may not increase the incidence of myelogenous neoplasms, but will induce other neoplasms. Our data suggest that exposure to 100 ppm benzene 6 hr/day, 5 days/week for 16 weeks may be close to such a time-dose threshold for myelogenous leukemias.

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