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THE IMPORTANCE OF PLURIPOTENTIAL STEM CELLS IN BENZENE TOXICITY

DAVID P. GILL, VERNON K. JENKINS, RENE R. KEMPEN and SYDNEY ELLIS

The Unwenity of Texas Medical Branch, Departments of Internal Medicine, Radiology and Pharmacology, Galveston, TX 77550 (U.S.A.)

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SUMMARY

Several schedules of benzene exposure were evaluated for their effects on peripheral white blood cell counts, bone marrow cellularity and transplantable colony forming units (CFU-S) in male C57 B1/6 mice. Intermittent exposure to 4000 ppm benzene in air produced leukopenia without altering the bone marrow cellularity. This same treatment, however, decreased the number of CFUS to 30% of control values. Uninterrupted exposure to lower levels of benzene decreased peripheral cell counts within 24 h, and later decreased marrow cellularity. Exposure of a non-dividing population of stem cells (CFU-S) to benzene for up to 24 h produced no detectable effect on the subsequent development of spleen colonies, suggesting that the effect of benzene on CFU-S occurs only after peripheral cells are depleted. These findings indicate that benzene has affects on both differentiated cells and undifferentiated stem cells. An effect on the pluripotential stem cell is an important aspect of benzene toxicity, but not its exclusive or initial site of action.

INTRODUCTION

Exposure to benzene has been implicated as the cause of Several types of blood dyscrasias in humans, including thrombocytopenia, granulocytopenia, anemia, pancytopenia and leukemia [1–3]. Involvement of the different hematopoietic cell lines suggest that a major target of benzene's action may be the pluripotential stem cell. Previous studies have indicated that the toxic effect of benzene is on cells in the early stages of differenti-

Address correspondence to: Dr. D.P. Gill, MW 404 R-3, University of Texas Medical Branch, Galveston, Texas 77550, U.S.A.

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ation, but not on stem cells [3,7]. However, other studies have shown that the number of stem cells in the bone marrow of mice was reduced after 3 days of exposure to benzene [8]. The latter data suggest that damage to these cells may be causally related to the development of hematologic abnormalities, including leukemia, following benzene exposure.

Stem cells are normally in a slowly dividing state [9,10] where they may not be affected by benzene. However, stem cells may enter a more rapidly dividing state in response to depletion of differentrated cells [9,11,12], and thus become more susceptible to injury. McCullough and Till [13] demonstrated that CFU-S transplanted into C57 B1 mice remain dormant in the spleen for 24 h after they are injected, and therefore recently transplanted stem cells are a model with which to examine benzene's effect on a nondividing population of CFU-S. During this initial 24-h period, the transplanted CFU-S demonstrate the Same sensitivity to ionizing radiation [13], and presumably other toxic agents, as CFU-S in unperturbed marrow.

In the present study several schedules of benzene exposure were used to assess the effects of benzene on peripheral blood cells, bone marrow cellularity, and hematopoietic stem cell numbers. In addition, the effects of benzene on a nondividing stem cell population (CFU-S) was studied.

MATERIALS AND METHODS

Mice and irradiation

Young adult C57 B1/6 male mice (20—25 g), obtained from Timco (Houston, TX) were used in these studies. The mice were maintained on standard laboratory chow and given water ad libitum. After total body radiation, the mice were caged under filter tops and were given sterile drinking water to reduce the risk of infection.

Recipients of bone marrow cells from benzene-treated donors or from untreated donors were given 800 rads total-body irradiation about 2 h before bone marrow injection to reduce their endogenous hematopoietic cells. Irradiation was delivered by a therapeutic ^{60}Co unit (C8 M/80, Picker Corp.) at a dose rate of 50 rads/min.

Benzene exposure

Mice were exposed to controlled concentrations of benzene in air in plastic cages fitted with plexiglass covers. Hardwood bedding and diet were provided as before treatment and changed every 2 days. The desired mixture of air and benzene was introduced into the chamber of approximately 2 liters/min, replacing the cage volume every 3—4 min. Exhaust gases from the cages were removed passively through 2 vent tubes extending from the plexiglass cover to the cage floor and the cages were kept in a laboratory hood during exposure for disposal of the benzene vapors.

Benzene vapor was prepared by passing compressed air through a fritted

glass gas dispersion apparatus (scrubber) immersed at a fixed depth in liquid benzene. The air leaving the apparatus, nearly saturated with benzene, was mixed with a second flow of compressed air to produce the desired final concentration of benzene. The combined flows were passed through a simple trap then into the exposure chambers. Air flow rates from each compressed air source were controlled with precision needle valves and flow meters.

Air samples were collected in gas tight syringes at the cage inlets for direct gas chromatography. Specimens were run isothermally on Propak Q at 130°C with a flame ionization detector. Solutions of benzene in methanol were used as standards.

Benzene treated donors were exposed intermittently to benzene at concentrations of 1000, 2000 and 4000 ppm in air, continuously or to benzene concentrations of 100, 500 or 1000 ppm. For intermittent exposures, the mice were exposed for 6 h each day, 5 days/week, for up to 6 weeks. During the remainder of the time, the mice were maintained in a conventional environment. For continuous exposure the mice were kept in a benzene and air atmosphere without interruption for up to 8 days. In one experiment bone marrow recipient mice were placed in a benzene atmosphere immediately after bone marrow cells were injected. Exposure to 100 ppm benzene was maintained for 12 h, 24 h, or continuously for the entire 8 days normally allowed for spleen colony development.

Blood wunts and spleen colony technique

Blood samples were obtained from the mice by amputation of the tip of the tail and 2–3 drops of blood were collected in siliconized micropipets. Total numbers of white blood cells were determined in an electronic cell counter (Accu-Stat®, Clay Adams) and differential counts were made by standard methods.

Assay of the number of hematopoietic stem cells in benzene treated mice was performed according to the spleen colony methods of Till and McCullough [14] as modified by Jenkins et al. [15]. Donor mice were killed by cervical dislocation, both femurs were removed, and the bone marrow cells were flushed out of the femurs in 1 ml of cold tissue culture medium. Aggregates of the bone marrow cells were dispersed by aspiration through a 23 gauge needle. The nucleated cells of the marrow suspension were counted in the electronic counter, and the number of nucleated cells/femur was determined. The cell suspensions of the various donor groups were diluted with tissue culture medium (TC 199, Difco®) to obtain a final concentration of 1×10^5 nucleated cells/ml. 0.5 ml of the cell suspension (5×10^4 cells) from each donor group was injected into the tail vein of irradiated recipient mice. The recipients were sacrificed 8 days later, and their spleens removed and fixed in buffered formalin.

The number of surface colonies of the spleens was determined under a dissecting microscope after the spleens were stained in Bouin's solution for 10–15 min. The mean number of surface colonies on the spleens of

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recipient mice was taken as the mean number of transplantable CFU-S obtained in 5×10^4 bone marrow cells of the donor mice.

RESULTS

Effects of benzene exposure on numbers of peripheml bbod cells

Intermittent exposures to benzene resulted in a concentration-related leukopenia that was evident on the third day of exposure, and reached the minimal number of white blood cells by the 5th or 6th day of exposure (Fig. 1). A concentration of benzene in air of 4000 ppm reduced the number of leukocytes to about 8% of the number in the same mice before treatment, whereas a concentration of 1000 ppm reduced the number of leukocytes to about 45% of pretreatment values (Fig. 1). Intermittent exposure to 4000 ppm benzene for up to 6 weeks resulted in sustained leukopenia. At this dose level the mice experienced tremulousness and were nearly immobilized during the 6-h exposure. These effects rapidly abated upon the return of the mice to room air and the mortality from continuing this treatment up to 6 weeks was negligible.

Differential blood counts were performed on animals exposed to 4000 ppm intermittently for up to 6 weeks. In control animals the lymphocyte count was $5.94 \pm 2.38 \times 10^3/\mu l$, and after counts stabilized during treatment lymphocytes were decreased to $1.34 \pm 0.44 \times 10^3$ cells/μl. Granulocytes were also decreased by this treatment. Control animals had $2.37 \pm 1.27 \times 10^3$ granulocytes/μl; during the course of benzene exposure, this fell to $0.5 \pm 0.17 \times 10^3$ cells/μl. The fall in both cell type was statistically significant ($P < 0.001$).

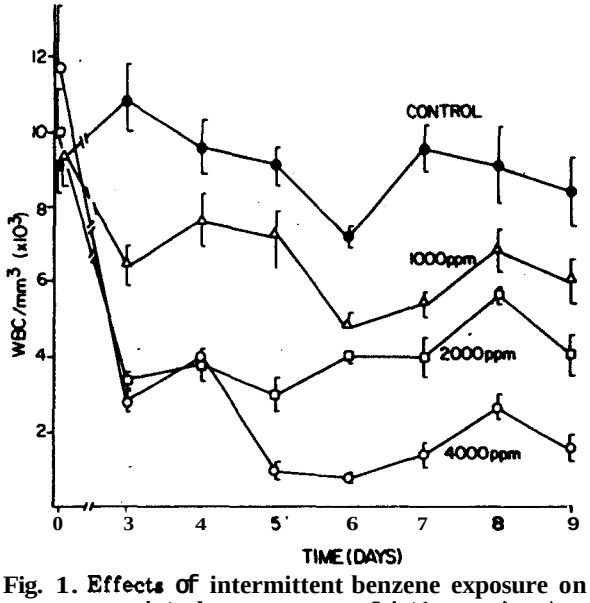


Fig. 1. Effects of intermittent benzene exposure on white blood cell counts. Animals were exposed to benzene vapor 6 h/day, 5 days/week. $n = 6$/group. Mean WBC ± S.E.

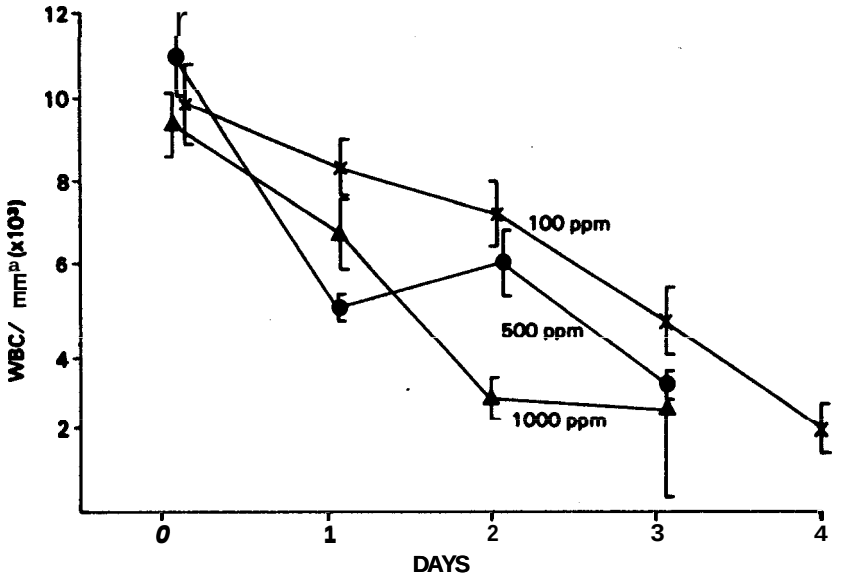


Fig. 2. Effects of continuour benzene exposure on white blood cell counts. Mean WBC ± S.E. For 100 ppm n = 6; for 500 ppm and 1000 ppm n = 12 on day 0 and day 1 n = 6 on day 2 n = 4 on day 3.

Continuous exposure of the mice to concentrations of 4000 ppm and 2000 ppm resulted in death of the mice within 24 h by causes unrelated to the hematopoietic system. Continuous exposures to 1000, 500 and 100 ppm were tolerated longer than the higher doses. However 1000 ppm and 500 ppm resulted in death of the animals after 3 or 4 days of exposure. Exposure to 100 ppm was tolerated for longer than 1 week. A decline in the number of peripheral white blood cells occurred in all 3 treatment

TABLE I

EFFECTS OF INTERMITTENT EXPOSURE TO 4000 ppm BENZENE IN AIR ON BONE MARROW CELLULARITY AND NUMBER OF TRANSPLANTABLE CFU-S[a]

	Weeks of exposure to benzene[b]				
	0	1	2	4	6
Mean number of cells} femur ± S.E. (x 10^6)	10.2 ± 0.4 (6)	9.2 ± 0.5 (6)	9.3 ± 0.5 (6)	10.0 ± 0.8 (6)	11.1 ± 0.5 (6)
Mean number of CFU-S 5 x 10^4 marrow cells ± S.E.	8.8 ± 1 (14)	3.8 ± 1.1[c] (4)	—	4.2 ± 1.4[c] (5)	2.2 ± 0.5[c] (15)

[a] Number of mice are given in parentheses.
[b] Mice were exposed to benzene 6 h/day 5 days each week.
[c] $P < 0.004$.

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groups within 24 h and reached values that were 25–30% of pre-treatment values by 3–4 days of exposure (Fig. 2).

Effects of benzene on bone marrow cellularity and *content of CFU*

Bone marrow cellularity of mice exposed intermittently to 4000 ppm benzene and air mixtures for up to 6 weeks was not altered (Table I). Even though bone marrow cellularity was not decreased, the number of spleen colonies (CFU-S) derived from 5×10^4 bone marrow cells of the benzene-exposed mice was reduced (Table I). After 1- and 4-week exposures, the numbers of CFU's were reduced to about 55% of the number of untreated donor mice and after 6 weeks of exposure the mean number of CFU's was reduced to 30%. A one way analysis of variance indicated that these were statistically different ($P < 0.0001$). Using contrast analysis each of the treated groups was determined to be statistically different from the initial value.

Bone marrow cellularity in mice exposed continuously to benzene at concentrations of 500 and 1000 ppm was not altered during the first 24 h, however, at the end of 48 h of exposure the cellularity was reduced to about 30% of the cellularity of mice before treatment (Fig. 3).

Effect of benzene on non-dividing stem cells

Exposure of recipients of 5×10^4 normal marrow cells to 100 ppm benzene for 12 h or 24 h after transplantation had no effect on the resultant number of spleen colonies. However, continuous exposure throughout the

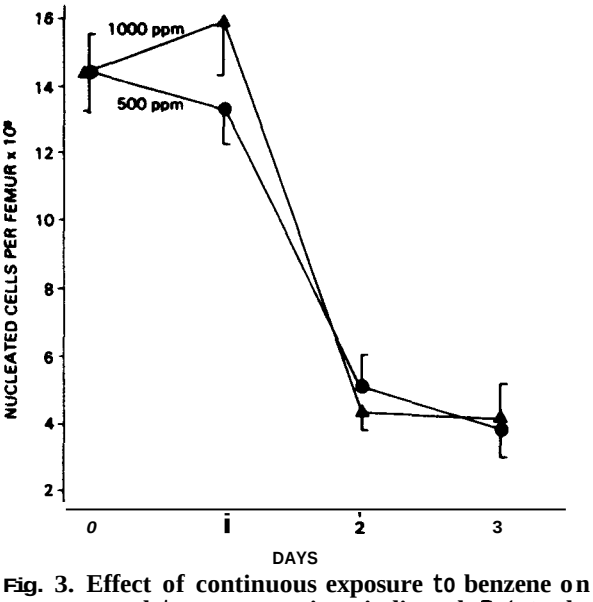


Fig. 3. Effect of continuous exposure to benzene on bone marrow cellularity. Animals were exposed to concentrations indicated. Points designate the mean number of nucleated cells/femur in millions ± S.E. Each **data point represents 8 femurs.**

TABLE II

EFFECT OF 100 ppm BENZENE IN *AIR* ON THE SURVIVAL OF TRANSPLANTED CFU-S. ANIMALS RECEIVED 5×10^4 NORMAL NUCLEATED MARROW CELLS

	Time of exposure to benzene			
	0 h	12 h	24 h	8 days
CFU-S/spleen mean ± S.E.	6.0 ± 1.1	5.6 ± 1.5	6.0 ± 1.0	0
Number of animals	5	5	5	4

8 day colony growth period, prevented development of the colonies (Table II).

DISCUSSION

In the present experiments, intermittent exposure of mice to benzene produced a rapid decrease in peripheral blood granulocytes and lymphocytes. The sensitivity of murine lymphocytes to benzene is not paralleled in humans, where granulocytes, megakaryocytes and red cells appear to be most vulnerable. However, our data does show that granulocytes are greatly reduced in C57 B1 mice by intermittent exposure to high levels of benzene. The CFU-S is clearly a precursor of granulocytes while the stem cell for lymphoid cells has not been clearly identified.

The number of CFU-S in the bone marrow of these animals is significantly reduced at 1 week, and remains decreased through 6 weeks of treatment. Uyeki [8] has previously shown that CFU-S are reduced in 3 days with similar intermittent benzene exposure. A decrease in CFU-S in the presence of normal marrow cellularity suggests that benzene may be selectively toxic to pluripotential stem cells. However, the normal marrow cellularity observed after intermittent treatment may result from an increased number of cell divisions or recovery from benzene's effect during the 18-h periods between benzene exposures.

If the stem cell is the primary site of benzene's effect, a reduction in stem cell numbers should be the first observable change. Later, marrow cellularity would decline because of inadequate entry of stem cells into the maturation pool. The peripheral white cells should remain normal in number until marrow storage pools of mature cells are depleted. Should the reverse be true, that is if toxicity occurs at a more differentiated cell population, then the pluripotential stem cells will be recruited from their usually slow cycle into a more rapidly proliferating state, and perhaps be rendered more susceptible to the toxic effects of drugs or radiation [9–11].

In order to ascertain the ultimate importance of stem cells in benzene's toxicity it was necessary to examine the sequence of the early hematologic

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events during the induction of toxicity. Continuous exposure to 100, 500 or 1000 ppm of benzene each produced a decrease in peripheral white blood cell counts within 24 h. This is almost certainly too early to reflect an effect on stem cells. By 48 h both peripheral counts and bone marrow cellularity were low which may indicate toxicity towards stem cells. However, this requires further investigation.

The effects of benzene were also studied on CFU-S in slow cell cycle, approximating the conditions in normal bone marrow. One hundred ppm benzene began to decrease peripheral white counts within 24 h (Fig. 2), but exposures of 12 h or 24 h had no effect on the subsequent development of spleen colonies by the stem cell population. Uninterrupted exposure to 100 ppm benzene continuously for 8 days completely inhibited spleen colony formation.

Our data indicate that pluripotential stem cells in the marrow are reduced early in the course of benzene poisoning but they are not the sole target for toxicity. CFU-S in a resting state are not affected by benzene, although, they are severely decreased in number by the time toxicity is well established. Whether injury to CFU-S occurs only when these cells begin to proliferate in response to losses of other cell types, remains to be demonstrated. An alternative explanation for decreased CFU-S in the marrow is that CFU-S migrate outside the marrow in response to benzene damage, and are therefore not as numerous in marrow specimens.

These findings may suggest possible explanations for several features of benzene toxicity. Isolated cytopenias are commonly reported in the literature [1,2,5]. In the presence of a markedly reduced stem cell compartment, the various hemopoietic hormones must compete for recruitment of the stem cells into committed lines of cell development [16,17]. Relatively minor variations in the hematopoietic factor activities or the local environment in the marrow may prevent adequate stem cell input into any one or several of the major marrow cell lines, which is ultimately manifested as an inadequate number of circulating blood elements.

A number of investigators [18,19] have found that stem cell differentiation ceases when the stem cell population falls to approximately 10% of its normal numbers. Cell division within the stem cell compartment under such conditions appear to produce only more stem cells and no cells committed to other cell lines. This phenomenon may explain the sudden onset of aplastic anemia during the course of chronic benzene intoxication, as this may occur when the critical stem cell population size is reached.

CONCLUSIONS

These experiments show that benzene has a relatively balanced effect on both differentiated cells and undifferentiatied stem cells in the hematopoietic system. An effect on the pluripotential stem cell is an important part of benzene's toxicity. Involvement of pluripotential stem cells is of theoretical importance to understanding the development of aplastic anemia and leukemia in response to benzene exposure.

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