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Benzene Hematotoxicity and Leukemogenesis

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Eight-to-twelve-week-old male and female C57B1/6 BNL mice were exposed to air or benzene vapor in air at a concentration of 10, 25, 100, 300, or 400 ppm. Benzene at concentrations of 100 ppm or higher for 10 exposures of 6 hours per day 5 days a week produced a reduction in bone marrow cellularity and the number of pluripotent stem cells in the bone marrow. The fraction of stem cells in DNA synthesis was also increased. Exposure to 300 ppm 6 hours a day 5 days a week for 2, 4, 8, and 16 weeks produced a diminution in the stem cell levels in bone marrow which returned to those of controls 2 weeks after benzene exposure for 2 and 4 weeks, 16 weeks after exposure for 8 weeks, and to 92% of controls 25 weeks after 16 weeks of exposure. There was a more rapid return of blood lymphocytes to the control level. Mice exposed to 300 ppm for 6 hours/day, 5 days per week for 16 weeks began dying at 330 days of age, whereas no deaths were observed in sham-exposed mice until 440 days of age. The benzene-exposed mice died in two waves: the first was from 330-390 days of age, with a second wave commencing at 570 days of age. The first wave of mortality was due primarily to thymic lymphomata. The second wave was due to a mixture of nonthymic lymphomata and solid tumors.

Key words: leukemogenesis, hemopoietic stem cells, benzene leukemogenesis, hemopoietic effects

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INTRODUCTION

Infante et al [1977] and Rinsky et al [1981] reported an increased incidence of

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Two males and two females with two tumors.

Leukemia in individuals who apparently were exposed to a time-weighted average of only 10 ppm. Further analysis of the concentration of benzene in the workplace showed that at times individuals could have been exposed to as high as 300 ppm. It is of major importance to determine if benzene concentrations at levels that produce no pancytopenia or perturbation of the hematopoietic stem cell pool will be leukemogenic. If this were the case, it would support the idea of a no-threshold linear dose response between benzene and acute myelogenous leukemia. The reported observations to date clearly indicate that further epidemiological studies and appropriate animal studies are essential to establish the dose-effect relationship between exposure to benzene, depression in hematopoiesis, and the incidence of leukemia. Accordingly, we have studied the effect of various concentrations of benzene on mice being exposed 6 hours/day for 5 days/week for 2 weeks to 16 weeks. Observations include blood counts, total cellularity of bone marrow, numbers of pluripotent stem cells in bone marrow, colony formation unit (CFU), fraction of the stem cells in DNA synthesis, the incidence of leukemia, and the development of solid tumors after cessation of exposure.

MATERIALS AND METHODS

Eight-to-twelve-week-old male and female C57B1/6 mice were exposed to conditioned air or air containing 10, 25, 100, 300, or 400 ppm benzene in chambers similar to those described by Hinners et al [1968], an isolation system described by Laskin et al [1970], or in glass "pickle-jar" chambers like those described by Leach [1965]. Mice were allowed food and water ad libitum except during exposure periods. Exposures were 6 hours/day, 5 consecutive days/week for 2 to 16 weeks. In a separate series of experiments, age-matched mice of the same strain were exposed to normal air or 300 ppm benzene 6 hours/day, 3 consecutive days/week for a total of 8 weeks.

At various times during and after the exposure periods, five-to-ten mice were removed from both the control and benzene-exposed groups, anesthetized by ether, bled by cardiac puncture via tail vein, and killed by cervical dislocation. Blood counts, differentials, hematocrits, and assays of CFU were performed 18-22 hours after termination of the exposure period or after a designated recovery period. White blood cell counts and red blood cell counts were performed on the blood specimens using a Coulter electronic counter. A hind leg was removed from each animal and the bone marrow was flushed from either the femur or the tibia and femur. In some cases the marrow was pooled, while in other studies mice were assayed individually. Total marrow cellularity was determined. Aliquots of some bone marrow suspensions were subjected to high specific activity, tritiated thymidine cytoide, in a modification of the method described by Becker et al [1965]. This procedure provides the percentage of stem cells in DNA synthesis. Briefly, cells ($1.5-2.0 \times 10^6/\text{ml}$) were incubated for 30 minutes at 37°C with $20 \mu\text{Ci}/\text{mmole}$ $^3\text{HTdR}$ at a concentration of $25 \mu\text{Ci}/\text{ml}$ ($0.3 \mu\text{g}/\text{ml}$ thymidine) or in a nontritiated thymidine solution of a similar concentration. After incubation the cells were washed in ice-cold buff containing excess thymidine ($30 \mu\text{g}/\text{ml}$) and resuspended in thymidine-free tissue culture medium. For each suspension (both cytoide and control for exposed and sham-exposed groups), recipient mice were fatally irradiated (730 or 750 Rads, 250 kVp X rays) 2-5 hours before being given 4×10^4 nucleated bone marrow cells intravenously to assay for the CFU content by the Till and McCulloch [1961] procedure. These recipients were killed 10

days after injection. Spleens were removed and fixed in Bouin's solution and the surface colonies were counted.

RESULTS

Figure 1 shows the effect of inhaling 0, 10, 25, or 100 ppm of benzene, 6 hours/day, 5 days/week, for 2 weeks (10 exposures) on the total number of nucleated cells obtained from a femur (marrow cellularity) as measured —20 hours after the final exposure. There was no effect of inhalation of 10 and 25 ppm, but there was a significant ($p < 0.003$, Student's *t*-test) depression in the marrow cellularity by inhalation of 100 ppm for 10 days over a 2-week interval.

Figure 2 shows the effect of inhaling 10, 25, 100, or 400 ppm of benzene, 6 hours/day, 5 days/week, for a total of 10 exposures over 12 days upon the CFU level in the femur expressed as percent of the age-matched controls. Ten and 25 ppm had no effect. There was a significant depression at 100 ($p < 0.001$) and a greater depression after inhaling 400 ppm ($p < 0.0004$).

Figure 3 shows the effect of inhaling 10, 25, 100, or 400 ppm of benzene for 6 hours/day, 5 days/week, for 2 consecutive weeks upon the fraction of hemopoietic stem cells (CFU) that are in DNA synthesis. The fraction in DNA synthesis was less than control-level after inhaling 10 ppm, but it was determined only once. There was no effect after inhaling 25 ppm, but there was a slight increase in the fraction in DNA synthesis after inhaling 100 ppm ($p < 0.17$) or 400 ppm ($p < 0.08$). These changes are of questionable significance. Previously, when 400 ppm was given over a longer time, a significant increase in fraction in DNA synthesis was observed [Cronkite et al, 1982].

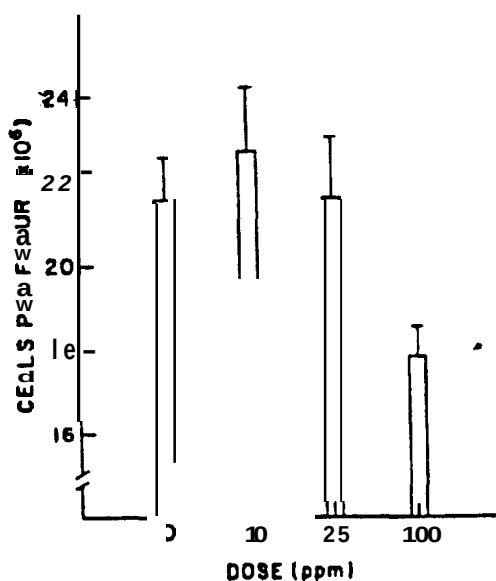


Fig. 1. The effect of inhalation of benzene 6 hours per day, 5 days per week, for a total of 10 exposures upon the marrow cellularity (total nucleated cells collected from one femur per donor). Mean $\pm$ S.E.M. shown, $n = 9$ to 10 per dose group.

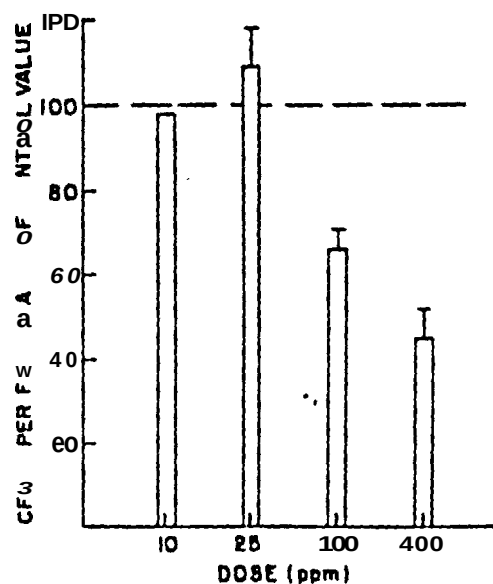


Fig. 2. The effect on CFU content of inhaling 0, 10, 25, 100, or 400 ppm benzene 6 hours per day for 5 days per week for a total of 10 exposures over 12 days. Mean $\pm$ S.E.M. shown. All data normalized to control value for each army. Three separate assays performed at 25 and 100 ppm, two assays at 400 ppm, and a single assay at 10 ppm.

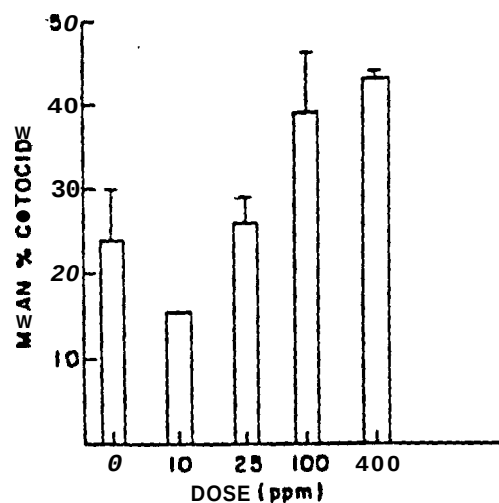


Fig. 3. The effect of inhaling 10, 25, 100, or 400 ppm of benzene for 6 hrs per day, 5 days per week for a total of 10 exposures upon the fraction of stem cells (CFU) in DNA synthesis expressed as percent cytocide by tritiated thymidine. Mean and S.E.M. shown. Three assays each at 25 and 100 ppm, two at 400 ppm, and one assay at 10 ppm.

After inhaling 10, 25, 100, or 400 ppm of benzene 6 hours/day, 5 days/week, for ten exposures over a 12-day interval, there was no significant effect upon the hematocrit at 10 or 25 ppm, but a significant diminution in the hematocrit was observed after exposure to 100 and a greater effect after exposure to 400 ppm (data not shown). The diminution can be explained in part on the basis of suppression in red cell production but probably also indicates hemolysis because sections of spleens of mice killed or dying during exposure periods were found to contain considerable deposits of hemosiderin.

Figure 4 shows the effect of inhalation of 10, 25, 100, or 400 ppm of benzene 6 hours/day, 5 days/week, for 10 exposures over a 12-day period upon lymphocytes and granulocytes. There was essentially no effect at 10 ppm, but thereafter a dose-effect relationship in the diminution of lymphocyte concentration in the peripheral blood was apparent. No effect on the granulocytes was seen throughout the exposure period.

Figure 5 shows the level of CFU (percent of the age-matched controls) as a function of time after exposure to 300 ppm for 2, 4, 8, or 16 weeks. After 2 or 4 weeks of exposure, the CFU had returned essentially to the level of the age-matched controls 2 to 4 weeks after the last exposure. After 8 weeks of exposure with the first assays being 4 weeks after the last exposure, the CFU was approximately 50% of the age-matched controls. There was recovery at 8 weeks, and by 16 weeks after the last exposure, the CFU level exceeded that of the age-matched controls. In the case of 16 weeks of exposure, the results shown are the means of two separate experiments. The first assay was 3 days after the last exposure, at which time the CFU level was approximately 27% that of the age-matched controls; however, 16 weeks after exposure the CFU level was still 60% of the age-matched control mice. In a single assay at 25 weeks after exposure the CFU level was 92% of control.

Figure 6 illustrates the effect on blood lymphocytes of inhaling 300 ppm of benzene 6 hours/day, 5 days/week, for 2, 4, 8, or 16 weeks. The top panel shows the

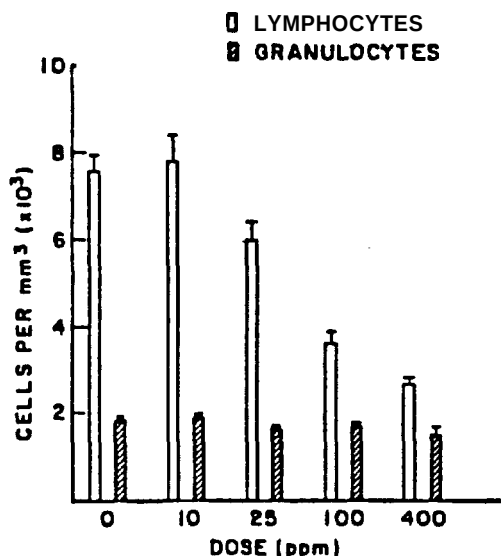


Fig. 4. The effect on blood lymphocytes and granulocytes of inhaling benzene 6 hours per day, 5 days per week for 10 exposures over 12 days.

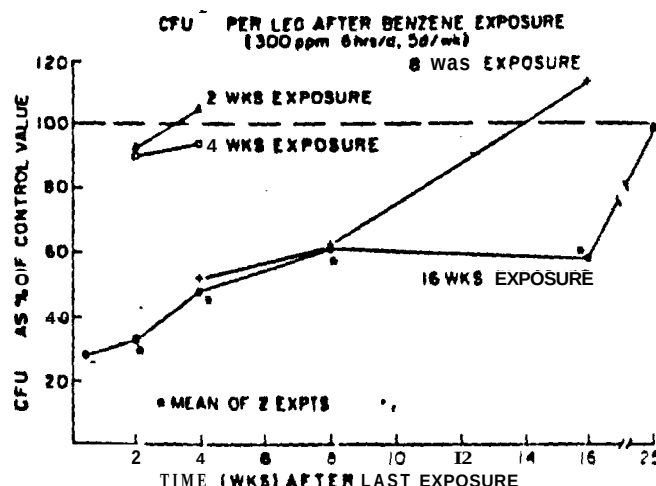


Fig. 5. The CFU levels (expressed as percent of age-matched controls) in mice that were exposed to 300 ppm, 6 hours per day, 5 days per week for 2, 4, 8, or 16 weeks and then observed until levels had returned to normal.

effects of 2 weeks of exposure, counts first being made 2 weeks after termination of exposure. At 4, 8, or 16 weeks postexposure, there was essentially no difference between the age-matched controls and the benzene-exposed mice. When mice were exposed for 4 weeks to 300 ppm, with the first counts being 2 weeks after the last exposure (second panel from the top), there was a significant difference between the benzene-exposed, and age-matched controls 2 and 4 weeks postexposure. At 8 and 16 weeks, this difference was no longer evident. After 8 weeks of exposure to benzene, the first counts were also made 2 weeks after the last exposure, at which time there was a significant difference ($p < 0.002$). By 8 and 16 weeks, there was no significant difference between the exposed mice and their age-matched controls. After 16 weeks of exposure, the first observations were made 3 days postexposure. At this time there was a marked difference between the benzene-exposed and the age-matched control mice ($p < 0.008$). For 8 weeks thereafter, there was a progressive increase in the lymphocyte levels in the blood of the benzene-exposed mice and at 8 and 16 weeks exposure there was no significant difference.

Figure 7 shows the cumulative mortality to date after mice were exposed to 300 ppm 6 hours/day, 5 days/week for a total of 16 weeks compared to sham-exposed mice. Benzene animals began dying at about 330 days of age, whereas no deaths were observed in the sham-exposed mice until 440 days of age. From 330 to 390 days there was a progressive increase in the cumulative mortality. The death-rate remained low until around 570 days of age, when a second wave of deaths commenced. Eight of the first ten mice that died had diffuse lymphomata with thymic involvement. A mixture of lymphomata and solid tumors, primarily of the zymal gland and ovaries, were found in mice dying during the later period. Table I lists tumor incidence by histological typing.

DISCUSSION

Our studies clearly show that 16 weeks of exposure to 300 ppm benzene, 6 hours/day, 5 days/week, produces a major diminution in the hemopoietic stem cells

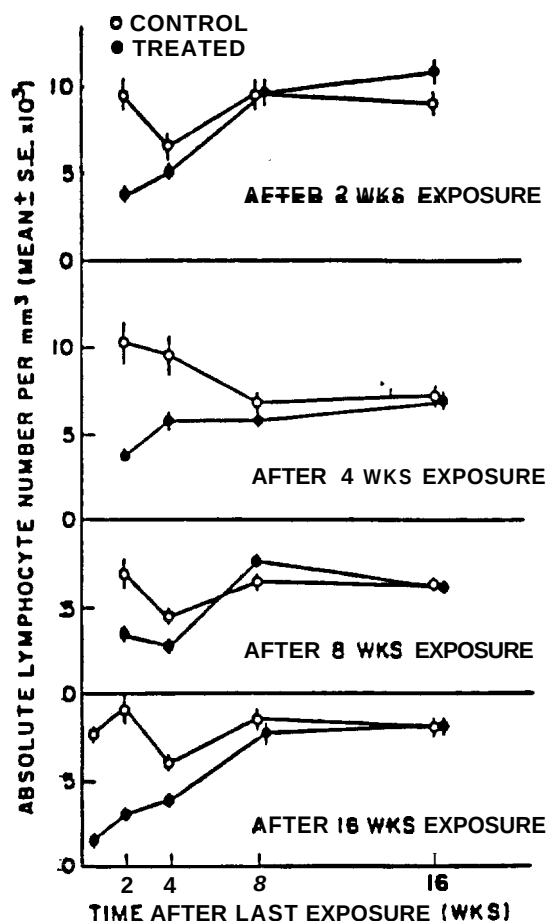


Fig. 6. The effect on blood lymphocytes of inhaling 300 ppm of benzene 6 hours per day, 5 days per week for 2, 4, 8, and 16 weeks and the regeneration after termination at exposure.

with incomplete recovery 16 weeks after termination of exposure. Whether recovery is ever complete is not known. In contrast to the CFU, lymphocyte levels in the peripheral blood return to normal levels. As reported previously [Cronkite et al, 1984], in the animals exposed for 16 weeks, there is a very significant increase in the incidence of leukemia that can be correlated with major destruction and incomplete regeneration of the hemopoietic stem cell pools. Whether leukemia will appear in mice in which there is no diminution in the hemopoietic stem cell pool remains to be seen. Others have reported effects of benzene on murine pluripotent stem cells. Gill et al [1980] found that exposure to 4,000 ppm benzene 6 hours/day, 5 days/week, reduced the CFU in bone marrow cells from 8.8 to 2.2 per 50,000 cells after 6 weeks of exposure. In contrast to our studies, they did not observe a decrease in bone marrow cellularity and did not calculate the total number of CFU per femur. Gill et al [1980] reported that 2 days of continuous exposure to 500 or 1,000 ppm resulted in a decrease in absolute marrow cellularity by 2 days, comparable to the decrease we observed after intermittent exposure.

Green et al [1981] exposed mice to graded doses of benzene (1.1 to 4862 ppm) for 6 hours per day for 5 days and measured the CFU content of spleen and femur 3

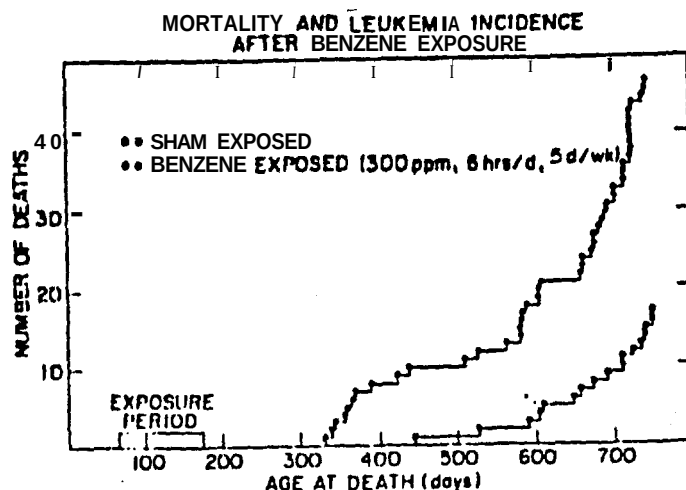


Fig. 7. Cumulative mortality after exposure of mice to benzene 300 ppm for 6 hours per day, 5 days per week for 16 weeks, compared to sham-exposed mice. The control population at risk totaled 88 mice and the exposed population 89 mice.

TABLE I. Tumor Incidence Among Sham and Benzene-Exposed C57 Female Mice*

	Sham-exposed	Benzene-Exposed
Effective No. at risk	88 (100) ^a	89 (100)
Leukemia (all types)	8 (9.1)	20 (22.5)
Lymphocytic neoplasm (thymic)	1 (1.1)	10 (11.2)
Lymphocytic neoplasm (nonthymic) ^b	2 (2.3)	6 (6.7)
Mycogenous leukemia	3 (3.4)	0 (0.0)
Leukemia (type not specified)	2 (2.3)	4 (4.5)
Zymbal ^c	1 (1.1)	16 (18.0)
Ovarian tumor	0 (0.0)	8 (9.0)
Hepatoma	1 (1.1)	1 (1.1)
Other neoplasm	2 (2.3)	4 (4.5)
Nonneoplastic lesion ^d	4 (4.5)	6 (6.7)
Surviving	65 (73.9)	41 (46.1)

*As of June 17, 1984. Total tumors exceed total number with tumor due to multiple tumor incidence.

^aNumber and percentage of at-risk population the latter shown in parentheses.

^bIncludes lymphocytic leukemia and solid lymphomas.

^cIncludes benign and malignant epidermoid tumor and lympho-epithelioma.

^dMice dead without neoplasm but with inflammation, hemopoietic distress, etc.

hours after the last exposure. At concentrations of benzene > 100 ppm, they observed a significant reduction in CFU content. Studies on recovery were not performed. Irons et al [1979] did not study stem cells directly; they did, however, examine the DNA content of the bone marrow cells and the incorporation of tritiated thymidine. Their studies showed a significant increase in cells in DNA synthesis, appearance of tetraploid cells, and a reduction in the specific activity of tritiated thymidine in DNA, suggesting an aberration in the cell cycle.

Uyeki et al [1977] exposed mice 8 hours/day to 4,680 ppm benzene for 1-, 3-, 3.5- and 8-hour sessions. They found reductions in the CFU and CFU content of bone marrow, but only measured the levels 24 hours after the last exposure, compared

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to our long-term studies of many weeks and our previous 65-day study [Cronkite et al, 1982].

Stoner et al [1980] exposed HRS/J mice of two genotypes—the leukemia-prone hairless mice (hr/hr) and the haired mice (hr/+) resistant to leukemia to 400 ppm benzene for 6 months commencing at 7 months of age. NO effect on the incidence of leukemia in either the (hr/hr) or (hr/+) was observed. Snyder et al [1980] exposed AKH mice to 100 ppm for 5 days per week for a lifetime. Then was no significant difference in incidence of lymphoma in controls or exposed mice. They also exposed C57B1/6J nude mice to benzene 300 ppm 6 hours/day, 5 days/week for a lifetime. Their observations on lymphoma induction ceased after about 500 days of exposure. At this time there was a significantly greater incidence of lymphoma in the benzene-exposed mice as compared to the controls. Our studies on C57B1/6J female mice exposed to the same concentration 6 hours/day, 5 days/week for 16 weeks induced a highly significant increase in the incidence of leukemia [Cronkite et al, 1984]. Our continuing studies showed a first wave of lymphoma commencing at about 150 days after exposure. A second wave of lymphoma and solid tumors commenced around 420 days after exposure. The present report shows the incidence up to 580 days after exposure. This exposure regimen also reduced the hemopoietic stem cell population significantly. Our studies show that exposure for 16 weeks to 300 ppm results in a significantly different pattern of lymphoma and solid tumors than has been observed after lifetime exposures. This suggests that continuous exposure either suppresses the incidence of lymphoma or shortens the lifespan of the mice, so lymphomas cannot be observed. This phenomenon may be similar to radiation carcinogenesis, where the incidence goes through a maximum as the radiation dose is increased.

Our studies suggest that lifetime or very prolonged exposure studies may be misleading. In chemical leukemogenesis it appears desirable first to determine a dose schedule that significantly decreases the hemopoietic stem cell pool without complete regeneration and then to determine if such a regimen is leukemogenic.

It is of major importance to determine if an exposure regimen that does not perturb hemopoiesis is leukemogenic. If it is not, we would suspect that there is a threshold concentration. If such a regimen remains leukemogenic, it would imply that there may not be a threshold.

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