

Experimental
Hematology

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The Hematopoietic Stem Cell Compartments in Mice during and after Long-term Inhalation of Three Doses of Benzene

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Abstract. Female BDF₁ mice were exposed for 16 weeks to airborne concentrations of 100, 300, and 900 ppm of benzene, 6 h per day, 5 days per week. Bone marrow hemopoietic stem cell compartments and peripheral blood cell counts were studied using clonal assays and standard methods. Dose-dependent depressive effects were observed on all stem cell compartments. Only the erythroid colony-forming units (CFU-E) compartment was depressed during exposures to 100 ppm; CFU-E were more sensitive than the erythroid burst-forming units (BFU-E), spleen CFU (CFU-S), or G-M CFU (CFU-C) during exposure to 300 ppm or 900 ppm. Lymphocytopenia was observed in the peripheral blood. After benzene-free intervals, a regeneration of lymphocyte numbers and slow normalization of stem cell numbers was seen. Complete recovery from the 16 weeks exposure to 300 ppm was seen between 73 and 185 days.

Key words: Hemopoietic stem cells — Mice — Benzene inhalation

Based on epidemiological data benzene is considered a human carcinogen [1-3]. Until recently there have been uncertainties whether the carcinogenicity could be demonstrated in experimental animals [4]. Inhalation studies with mice have shown development of tumors in a high percentage of exposed animals [5]. The toxicity of chronic benzene exposure to hemopoietic stem cells and the effects on some functions of the immune system have also been described [6-12]. The doses used in the previous laboratory studies varied between 10 and 4000 ppm. Our research program with benzene focused on the different hemopoietic stem cell types and addressed questions related to long-term exposure to concentrations of 100, 300, and 900 ppm. The objectives were to identify the most sensitive parameter and to determine if a dose-response effect could be demonstrated.

Materials and methods

Animals. Female mice (21-25 g) were used (C57Bl/6 × DBA/2 F₁ hybrids). These were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, FRG. They were fed commercial pellets and water ad libitum. During the benzene exposure period of 6 h, no food was made available. The mice were kept under controlled climatic conditions in the Central Animal Facility of the University of Ulm.

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Inhalation exposure. Benzene vapor was generated by forcing filtered air through a box with a constant influx of benzene. The air stream containing benzene was mixed with a secondary flow of air to obtain the desired mixtures. The inhalation chambers provided 20 air changes per hour. Benzene concentrations in the chamber were monitored twice daily by gas chromatography as described by Zinseler [13]. The variance was <5% deviation from the desired concentration. The mice were exposed to the benzene mixtures for 6 h per day, 5 days per week (Monday through Friday). Control mice were housed in the same chambers without admixture of benzene.

Hematological techniques. Blood samples were obtained from the axillary plexus after ether anesthesia. Total numbers of white blood cells were determined using an electronic cell counter (Coulter Counter ZF). Differential counts of blood smears and the reticulocyte counts were obtained using standard methods. Organ weights of the spleen and the thymus were also determined.

Bone marrow cells from the femur were flushed out into a known volume of medium, suspended in a single-cell suspension, counted, and subsequently used for the stem cell assays. Spleen colony-forming units (CFU-S) were determined using 800-rad irradiated recipients (x-rays, 280 kV, 12 mA with 1.5 mm Cu and 1.0 mm Al as filter, focal distance 70 cm, 30 rad/min). Macroscopic spleen colonies were counted at day 9 without further characterization.

Erythroid burst-forming unit (BFU-E), erythroid colony-forming unit (CFU-E), and G-M CFU (CFU-C) cultures were established in methylcellulose using standard protocols as described in previous communications from this laboratory (see Seidel and Opitz [14] and Seidel and Kreja [15]). Cultures were established using fetal calf or horse serum. Mixed-lymphocyte-culture supernatant was used as a source of burst-promoting activity for the BFU-E cultures. Post-endotoxin serum from mice was used as a source for colony-stimulating activity in the CFU-C cultures. Sheep erythropoietin step III was used for BFU-E and CFU-E cultures. All colony counts were converted into colony numbers per femur. Each day control cultures were set up using the pooled femoral marrow of three control animals. At all experimental points four benzene-exposed mice were tested individually and their data were compared with those of the controls. Unless indicated otherwise, the assays were done on Monday mornings, i.e., about 66 h after the end of the exposure period of the preceding week. For the data summarized in the tables and figures, the colony numbers of the controls were set as 100%. The absolute numbers per femur were as follows: CFU-S — 5000, CFU-C — 28,000, BFU-E — 8000, and CFU-E — 30,000.

Results

Stem cells during the exposure period

The relative numbers of BFU-E, CFU-E, and CFU-S per femur in mice exposed to 100 and 300 ppm for 16 weeks and to 900 ppm for 8 weeks are shown in Figure 1. There was considerable variation. Table 1 summarizes the data. A dose-response relationship can be seen, especially after 8 weeks of exposure for the BFU-E and CFU-E. Also, the CFU-E compartment seems to be more sensitive than the BFU-E and the CFU-C compartments. This is especially the case

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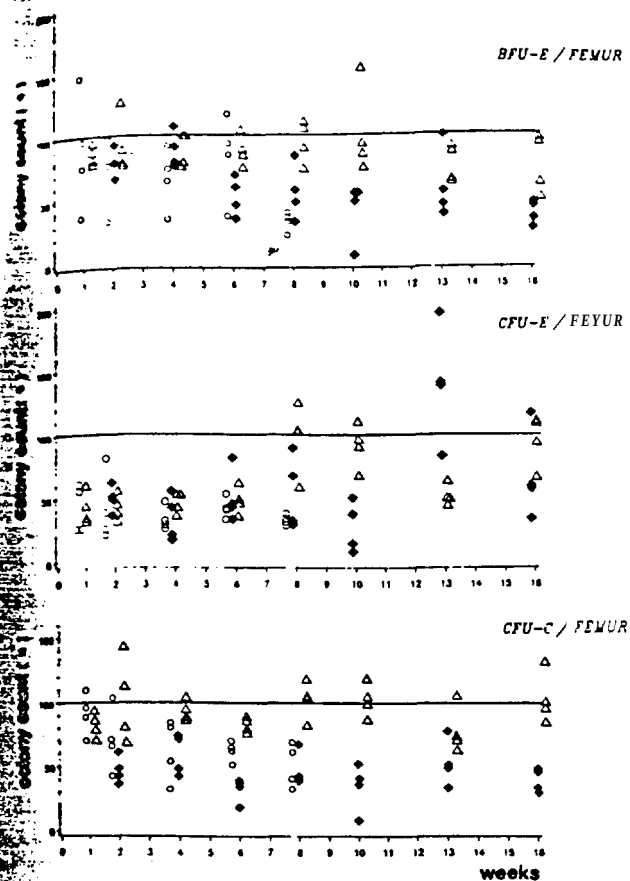


Fig. 1. BFU-E, CN-E, and CN-C numbers per femur (as percent of controls) in individual mice exposed to 100 ppm (A), 300 ppm (B), and 900 ppm (C) of benzene.

for the mice exposed to 100 ppm, for which no effect was seen with the BFU-E at least up to 10 weeks. The CFU-E — below 60% from 1 week onwards. The CFU-C compartment does not seem to be affected as much as the CFU-E. The same holds true for CFU-S (Table 1). For the group with a 300-ppm exposure, after 13 weeks the CFU-E numbers were above the control level for three out of four animals.

Stem cells after the exposure period

After an exposure period of 8 weeks animals were evaluated after benzene-free intervals. Figure 2 presents the BFU-E and CFU-E determinations. Ten and 17 days after 100-ppm ex-

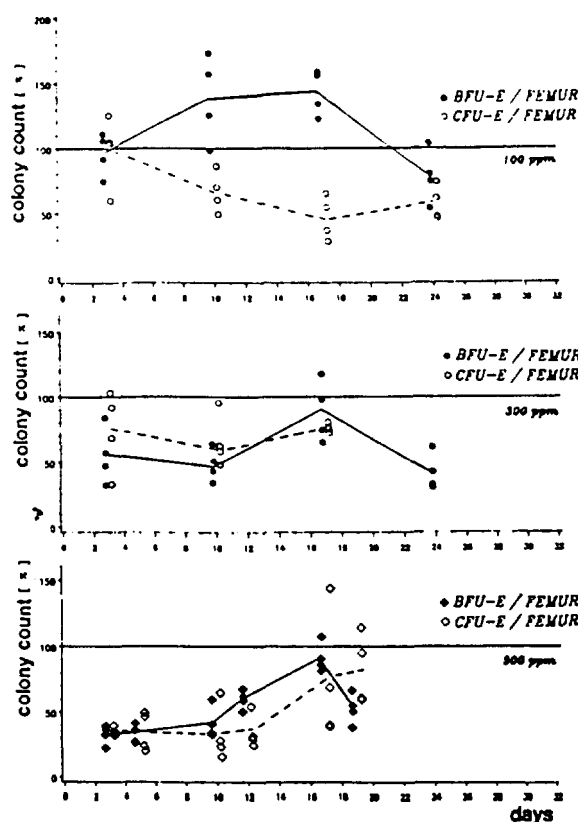


Fig. 2. BFU-E (closed symbols) and CFU-E (open symbols) numbers per femur (as percent of controls) in individual mice after previous exposure over a period of 8 weeks to 100 ppm, 300 ppm, and 900 ppm, respectively, of benzene. Lines combine mean values.

posure, the BFU-E were above control values and the CFU-E below control values, although normal levels were seen at day 3 (66 h after the end of the exposure). After an exposure to 300 and to 900 ppm, a slow regeneration of the stem cell numbers was seen. The BFU-E data at day 24 after a 300-ppm exposure, and at day 19 after a 900-ppm exposure, seem to indicate that a steady state was not reached after this time period.

The stem cell recovery after 300 ppm of benzene exposure over a period of 16 weeks is shown in Figure 3. There was a wave of regeneration with an overshoot of CFU-E during the first 3 weeks in a normal atmosphere. Most BFU-E and CN-E numbers remained below controls at least for 73 days: a complete normalization was achieved by day 185.

Table 1. Stem cell numbers in femurs of benzene-exposed mice as percent of controls (mean $\pm$ SD)

	4 weeks		8 weeks	
	BFU-E	CFU-E	BFI-E	CFU-E
100 ppm	88% $\pm$ 13%	48% $\pm$ 8%	96% $\pm$ 17%	99% $\pm$ 28%
300 ppm	88% $\pm$ 14%	35% $\pm$ 18%	55% $\pm$ 22%	73% $\pm$ 30%
900 ppm	66% $\pm$ 25%	35% $\pm$ 16%	32% $\pm$ 8%	35% $\pm$ 2%
	CFU-S	CFU-C	CFU-S	CFU-C
100 ppm	92% $\pm$ 11%	94% $\pm$ 8%	ND	103% $\pm$ 15%
300 ppm	70% $\pm$ 19%	59% $\pm$ 17%	65% $\pm$ 15%	47% $\pm$ 13%
900 ppm	82% $\pm$ 17%	63% $\pm$ 24%	ND	51% $\pm$ 17%

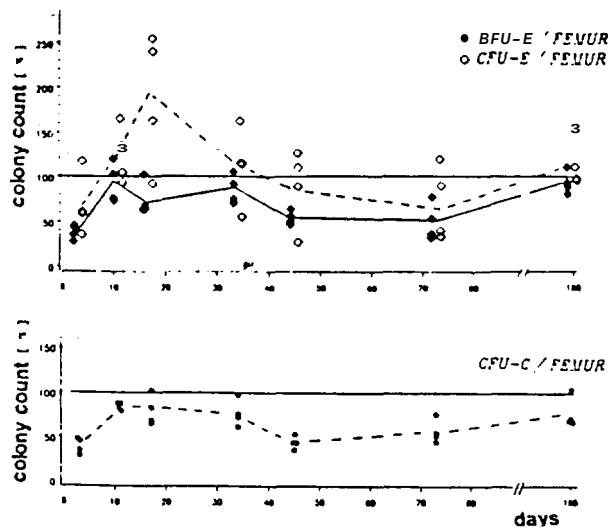


Fig. 3. BFU-E and CFU-E (upper part) and CFU-C (lower part) numbers per femur (as percent of controls) in individual mice after previous exposure over a period of 16 weeks to 300 ppm of benzene. Lines combine mean values.

Peripheral blood

A slight anemia developed in mice exposed to 300 and to 900 ppm of benzene. After 4 weeks the erythrocyte count was $7.4 \times 10^6/\text{mm}^3$ and $7.6 \times 10^6/\text{mm}^3$ as compared with $9.5 \times 10^6/\text{mm}^3$ in controls. The anemia did not develop further during the next weeks. With 300-ppm exposure a slight recovery, even during the benzene exposure period, was seen to reach $8.6 \times 10^6/\text{mm}^3$ at 13 weeks and also at 16 weeks. The reticulocytes showed considerable variation. Three out of four animals showed a reticulocytosis at 13 weeks. The same animals had elevated numbers of C N - E in the marrow (see Fig. 2). Also, three out of four animals had elevated reticulocyte numbers 2 weeks after the end of the 16-week exposure period. The number of granulocytes was not affected by the benzene exposure.

The number of lymphocytes showed a dose-dependent reduction. As shown in Table 2, there were high lymphocyte numbers in the control group of the 300-ppm experiment, which had been performed 1 year earlier. There is no explanation for this difference. With a 100-ppm exposure, the number of lymphocytes is normal immediately after the end of the exposure (66 h; see above), but there is a decline after 1 or 2 weeks in a normal atmosphere. The effect on the lymphocytes in the 300- and 900-ppm groups was reversible; in most instances a benzene-free interval of 1 or 2 weeks was enough for complete regeneration or even an overshoot.

Discussion

There are several reports of studies on hemopoietic stem cells and peripheral blood in benzene-exposed mice. These studies showed substantial effects. A dose-dependent CFU-S reduction after 10 exposure days (6 h per day) was reported by Cronkite et al. [9] with doses up to 400 ppm that were inversely related to the increase of CFU-S in DNA synthesis. Gill et al. [7] reported a depression to about 20% after 4000 ppm over a period of 6 weeks. Depressed CFU-

Table 2. Number of lymphocytes in the peripheral blood of benzene-exposed mice

	Exposure period	Time in normal atmosphere	
	4 weeks	1 week	2 weeks
Control	2990 ± 1150 ^a	—	—
100 ppm	2690 ± 1580	1860 ± 320	—
Control	4800 ± 2000 ^a	—	—
300 ppm	2230 ± 380	3130 ± 900	3750 ± 1250
Control	2570 ± 750 ^a	—	—
900 ppm	1270 ± 590	7390 ± 1070	4010 ± 1040
	8 weeks	1 week	2 weeks
Control	3170 ± 1700 ^a	—	—
100 ppm	3510 ± 1120	1780 ± 240	2080 ± 550
Control	6500 ± 1000 ^a	—	—
300 ppm	2170 ± 270	3370 ± 500	3690 ± 900
Control	3070 ± 820 ^a	—	—
900 ppm	1250 ± 460	3660 ± 1670	—

Peripheral blood lymphocyte numbers in mice exposed to concentrations of benzene, studied at the end of the exposure period and after 1 and 2 weeks in normal atmosphere.

^a Numbers obtained from three groups of control mice: immediately after the exposure period (4 and 8 weeks, respectively) and 1 and 2 weeks later. Their numbers were matched ($n = 9$). Mean values ± SD.

C numbers were reported by other researchers [6, 7, 10]. Hilderbrand and Murphy [10] presented BFU-E and CFU-E data for an exposure to 4062 ppm over 14 days. In their study an initial depression of CFU-E (deeper than BFU-E levels) was observed however, during the benzene exposure the C N - E rebounded to slightly higher levels than the control values. This might, in principle, be the same phenomenon as seen in our study for a benzene exposure of 300 ppm at 13 weeks. The lowest concentration of benzene that was studied was 10 ppm. This was for an exposure period of 173 days conducted by Baarson et al. [11]. They reported a depression of CFU-E below 5% of controls but not of BFU-E. It must be noted that these studies were different with respect to the strain of mice used, in vitro techniques used, and also doses and time intervals studied. Our present study will allow a better evaluation and comparison of the effects, because three different assay systems (four when CFU-S were included) were used simultaneously. Identical protocols in the three experimental runs were used.

We concluded that the erythroid system, as represented by the BFU-E and the CFU-E in the marrow and the erythrocytes in the peripheral blood, is more sensitive than the myeloid cell system. This holds true when evaluations are made using CFU-C numbers and peripheral blood neutrophils. Within the erythroid system the CFU-E are the most sensitive—in agreement with previous reports cited above. There are, however, important observations that warrant further investigation. The high CFU-E number observed late in the exposure period, accompanied by a reticulocytosis, must be explained. Also, our observation that the anemia did not worsen during the exposure periods may indicate that the erythroid cell system can adapt to the benzene; this should be evaluated. This aspect will be described in future communications.

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In the experiment with 100 ppb, a depression of CFU-E numbers was seen as the principal effect of benzene exposure. After 8 weeks of benzene exposure with a subsequent short exposure to periods of normal air, an overshoot of BFU-E numbers and a lymphocytopenia were observed. This may indicate the presence of regulatory processes that should be evaluated. The other effects observed on lymphocyte numbers were in agreement with the data of Cronkite et al. [5]. The doses used in our studies are high when compared with human exposure data and threshold values suggested by various organizations (see [16]). Therefore, concentrations of 100 ppm should be studied. The CN-E data of Baarson & [11] for 10-ppm exposures and other experiments with mice and rats utilizing cytogenetic methods indicate that effects can be expected [17]. It should be possible to compare the sensitivity of the various cell compartments and evaluate the techniques for their appropriateness in establishing a "no effect" level for benzene exposure.

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