EFFECTS OF BENZENE INHALATION ON MURINE PLURIPOTENT STEM CELLS

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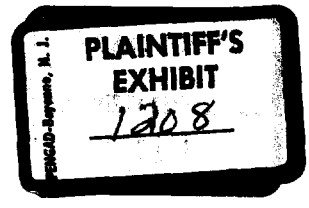

Effects of benzene inhalation on mouse pluripotent hematopoietic stem cells have been evaluated. Male mice 8-12 wk old were exposed to 400 ppm benzene for 6 h/d, 5 d/wk, for up to 9½ wk. At various time intervals exposed and control animals were killed, and cardiac blood was evaluated for changes in white blood cell (WBC) and red blood cell (RBC) content. In addition, femora and tibiae were evaluated for total marrow cellularity, stem cell content (as measured by the spleen colony technique), and the percent of stem cells in DNA synthesis (as determined by the tritiated thymidine cytocide technique). Exogenous spleen colonies grown from marrow of exposed animals were counted, identified, and scored by histological type. Exposure to benzene caused significant depressions of RBCs and WBCs throughout the exposure period, which continued for at least 14 d after exposure. Bone marrow cellularity and stem cell content were also depressed in exposed animals throughout the study. Tritiated thymidine cytocide of spleen colony-forming cells was generally increased in exposed animals, perhaps indicating a compensatory response to the reduction of circulating cells. Spleen colonies of all types were depressed after exposure to benzene. The significance of the reduction in cellularity, stem cell content, and changes in morphology of spleen colonies is discussed in relation to cellular toxicity and residual injury.

INTRODUCTION

The biochemical and morphological changes observed in identifiable hematopoietic cells are phenotypic changes. Irrespective of the cause, they result from genotypic alterations in the pluripotent stem cell that perpetuates hematopoiesis. Although of general interest and useful for diagnosis or detection of toxicity, phenotypic changes are of little value in understanding the mechanism of a pollutant effect. Accordingly, in our studies of hematotoxicity we concentrate on the hematopoietic stem cell, its rate of proliferation and differentiation into identifiable cell lines, and

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the mitotic potential of the immediate descendants of the pluripotent stem cell.

The pluripotent stem cell can only be assayed in the mouse by counting the number of gross spleen colonies formed in the fatally irradiated mouse following injection of a known number of bone marrow cells (Till and McCulloch, 1961). The cell forming colony is called the colony forming unit in spleen (CFU-S). The colonies are clones arising from a single cell. After several self-renewing mitoses, the colonies commence to differentiate down the red, white, megakaryocytic, or mixed cell lines. After 5–10 d, the colonies are easily seen by eye on the surface of the spleen.

The hematologic phenotypic alterations induced by benzene have been reviewed (Laskin and Goldstein, 1977; Snyder and Kocsis, 1975). They indicate injury in self-renewing CFU-S that is transmitted to the identifiable progeny. The injury may be manifested by a permanent change in genotype, in which case the phenotypic expression will be permanent, Disappearance of the phenotypic expression is indicative of either repair of the genotypic injury by processes yet to be identified or dying out of the affected clone of stem cells.

Uyeki et al. (1977) exposed mice 8 h/d to **4680** ppm benzene for 1, 3, and $3\frac{1}{2}$ 8-h sessions and then measured the cellularity, CFU-S, and CFU-C content of bone marrow. Colony forming units in culture (CFU-C) are believed to be the progenitor cells of granulocytes and macrophages. They are detectable by their ability to form macroscopic colonies of granulocytes and/or macrophages in agar culture. One 8-h exposure reduced the CFU-C content of marrow to 37% of the control value the day after the exposure without any change in cellularity. The **CFU-C** progressively increased on d 4 and 7 but was still **23%** below controls. Three and one-half 8-h exposures resulted in further decreases in CFU-C content of the marrow. Three 8-h exposures dropped the CFU-S content of marrow by nearly a factor of 3, measured 24 h after the last session. The concentration of benzene was much greater than in our studies and observations were made for only a few days, compared to our 65-d study.

The studies described above involved benzene treatment schedules that bear little relation to the workplace. In this paper we present data on exposure of mice 6 h/d, 5 d/wk, at a concentration of benzene known to produce hematologic effects. The focus of our study in this initial work will be on the pluripotent stem cell, in which continuing or permanent injury to genetic control of hematopoiesis would reside. In future studies we will address dose, time, and effect relations.

METHODS **AND** MATERIALS

Benzene Exposure

Male Hale Stoner BNL mice 8–12 wk old were exposed to either normal air or 400 ppm benzene in chambers for up to $9\frac{1}{2}$ wk (6 h/d, 5

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d/wk). Exposures to benzene were carried out in an isolation chamber system similar to that described by Laskin et al. (1970). Mice were housed in nesting boxes, kept in the living quarters of the multicompartmental chamber, and were allowed food and water *ad libitum*, except during the exposure periods. The room was maintained on a 12-h light cycle. During exposure animals were placed in wire cages and then put into a 128-l exposure chamber mounted inside the isolation compartment. Control animals were housed in similar cages for exposure to air only. The chamber flow was maintained at approximately 30 l/min. Benzene vapors were generated by metering air through a bubbler and then into the airstream of the inhalation chamber. Concentrations were monitored at $\frac{1}{2}$-h intervals by using an automatic gas sampling system coupled to a gas sampling loop of a Packard model 417 gas chromatograph. The chromatographic data were confirmed by wet chemical analysis for benzene vapors, using a bubbler with ethanol as the absorbing solution. Absorbance was measured at a wavelength of 254.6 nm with an ultraviolet spectrophotometer.

At various times during and after the exposure period two to four mice were removed from both the control and benzene groups, anesthetized by ether, bled by cardiac puncture, and killed by cervical dislocation. Blood counts and assays for CFU-S were performed 18 h after termination of the exposure period or at the time designated in the Results section after exposure had been terminated. White blood cell (WBC) and red blood cell (RBC) counts were performed on the blood specimens by Coulter electronic counting. For WBC counts, the red cells were lysed by Zap-Isoton II* (Coulter Diagnostics, Hialeah, Fla.) and then counted. The red cells were counted in blood diluted 1:50,000 in Isoton II. Since blood counts of experimental animals are expressed as percentages of control values, the means (± SE) for control animals are: RBC, $7.56 \pm 0.13 \times 10^6$, and WBC, $5.9 \pm 0.3 \times 10^3$. It is to be noted that control animals are bled at each time and results for the experimental animals expressed as a percentage of the average value at that time.

The hind legs were removed and the bone marrow was collected from the pooled tibiae and femora by the grinding method (Stoner and Bond, 1963). Total marrow cellularity was determined from the resultant suspensions. An aliquot of each bone marrow suspension was subjected to tritiated thymidine ([^{3}H]TdR) cytocide (Becker et al., 1965). For each suspension (both cytocide and control for exposed and sham-exposed groups) 25 Hale Stoner BNL mice (8–12 wk old males) received 750 rads from a 250-kVp, 30-mA GE Maxitron X-ray machine with 1-mm Al and 0.5-mm Cu filtration at a dose rate of 110 rad/min, 2 h before being given 60,000 nucleated bone marrow cells iv to assay for CFU-S content (Till and McCulloch, 1961). These recipients were killed 7 d after injection. Their spleens were removed and fixed in Bouin solution and the surface colonies counted. Selected spleens were embedded in paraffin, serially sectioned at 5 μm, and stained with hematoxylin and eosin. All colonies

were identified and classified as to histological type following the criteria of Reincke et al. (1976).

RESULTS

The results reported below represent 1–47 exposures for **6** h/d, 5 d/wk, to about **400** ppm benzene. Daily averages ranged from **346** to 438 ppm, with the average concentration for any subgroup of animals ranging from **389** to **423** ppm. The cumulative average concentration for the entire series of exposures was 399 ppm.

Changes in RBC counts are shown in Fig. 1. On d 11 of the study, after 9 exposures to **400** ppm benzene, there was a significant decrease in the RBC count, which remained between 54 and 80% of the control value. The oscillations may represent changes in the rate of destruction or production of the RBCs or degree **of** hydration of the mice. After **65** d **(47** exposures) benzene exposures were terminated. Although the **RBC** count increased during **14** d after the termination of exposure, it was still 91% of the control value.

Changes in total WBCs are shown in Fig. 2. There was *a* precipitous drop in the WBC count to **35%** of the control value by d **4** of exposure. There were large fluctuations in the total WBC count, which are not yet understood. Following the termination of benzene exposure, the WBC count progressively increased from **38** to **80%** of the control value within 14 **d.**

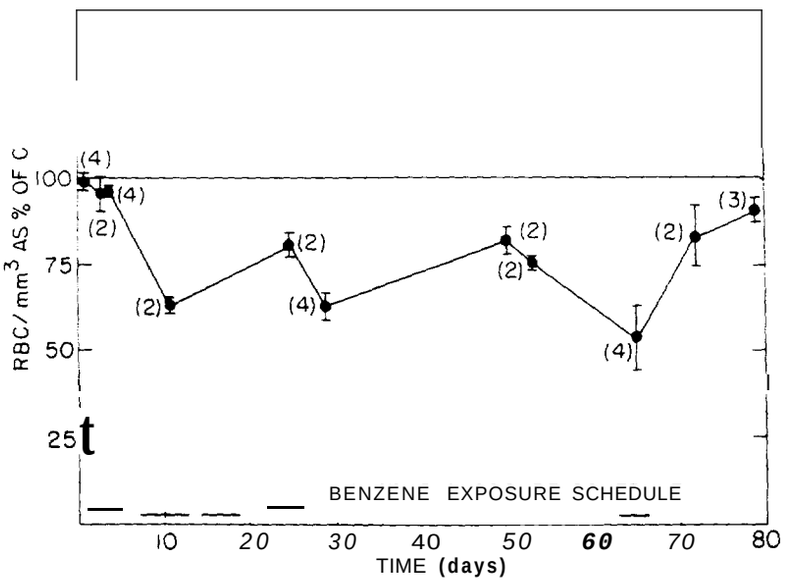
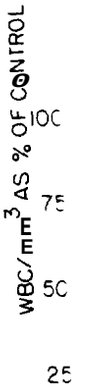


FIGURE 1. Serial **RBC** counts on **mice exposed** *to* **benzene. Numbers in parentheses are numbers of mice studied. Data points are means ± SE.**



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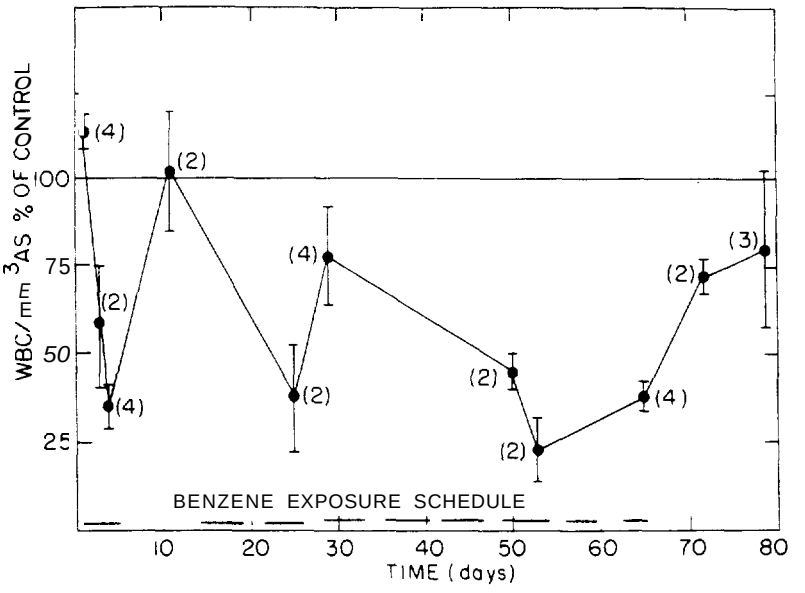

FIGURE 2. *Total WBC count of mice exposed to benzene. Numbers in parentheses are numbers of mice studied. Data points are means ± SE.*

Total bone marrow cellularity was determined on pooled marrow from femora and tibiae of two to four mice (Fig. 3). Error bars are not shown since the counts were made on pooled bone marrow. The total bone marrow cellularity decreased.to 33% of the control value by the 5th exposure day and remained at this level through the 25th day of study. Except for d 50, when the cellularity of exposed mice was equal to that of controls, bone marrow cellularity remained at about 50% of control values until benzene exposures were terminated. After the termination of benzene exposures, the marrow cellularity increased promptly, to 88% of the control value 14 d after the last exposure.

Changes in the CFU-S content of the pooled marrow suspensions are shown in Fig. 4. Twenty-five recipient mice were used to assay each pooled marrow sample. After 5 d of exposure, the CFU-S content of the femora and tibiae had dropped to 23% of the control value. Thereafter there were oscillations in the CFU-S content, which ranged between 13 and 43% up to 14 d after termination of exposure. At 1, 5, and 11 d, the studies were repeated and the results were comparable.

Serial changes in the percent of CFU-S killed by exposure to high doses of [^{3}H]TdR are shown in Fig. 5. The percent of [^{3}H]TdR cytocide is a close approximation to the fraction of CFU-S actively synthesizing DNA. Thus 5–25% of the CFU-S of control animals were in DNA synthesis. CFU-S in DNA synthesis in benzene-exposed mice decreased from 26 to 13% during the first 3 d of exposure, increased progressively

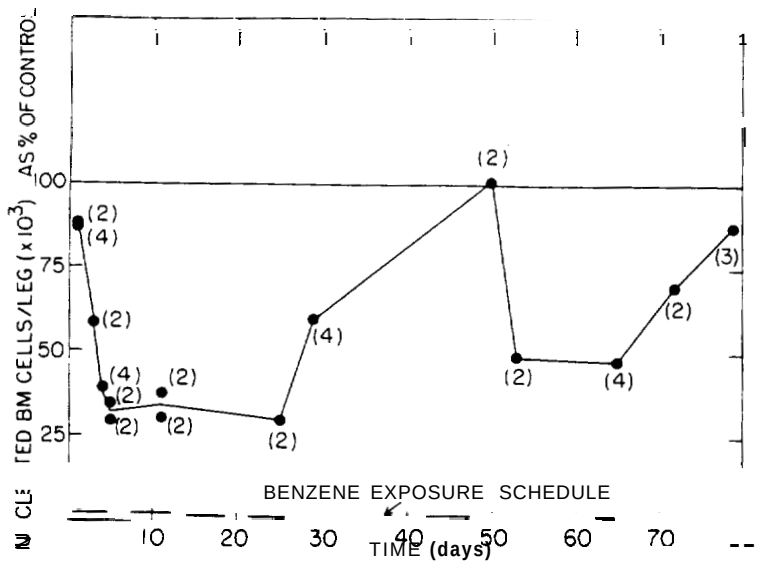

FIGURE 3. Serial changes in total number of nucleated cells per leg (femur plus tibia) of mice exposed *to* benzene. Where there are two data points they represent separate studies. Numbers in parentheses are numbers of mice studied. Marrow is pooled from animals killed on a given day.

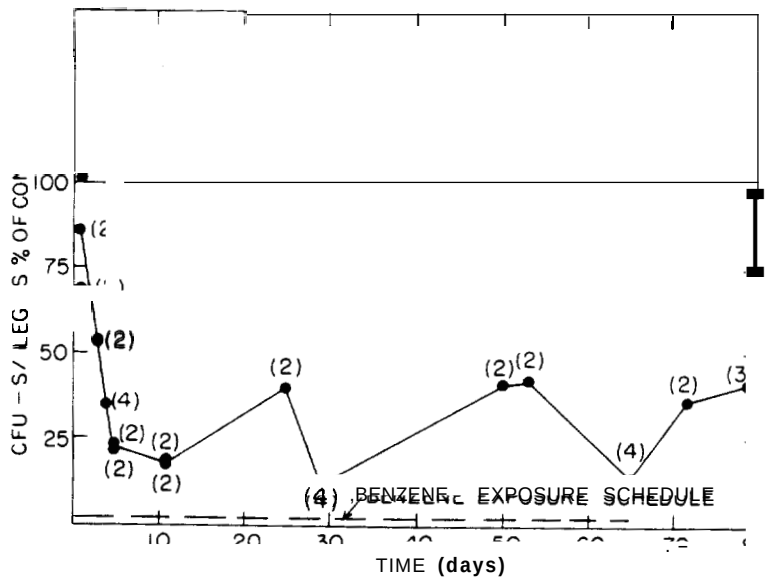

FIGURE 4. Serial studies of the number of **CFU-S** per leg (femur plus tibia) while mice are exposed to benzene. The **CFU-S** assay is performed on the pooled bone marrow by injecting 6 X 10^4 bone marrow cells iv into the 25 fatally irradiated recipients.

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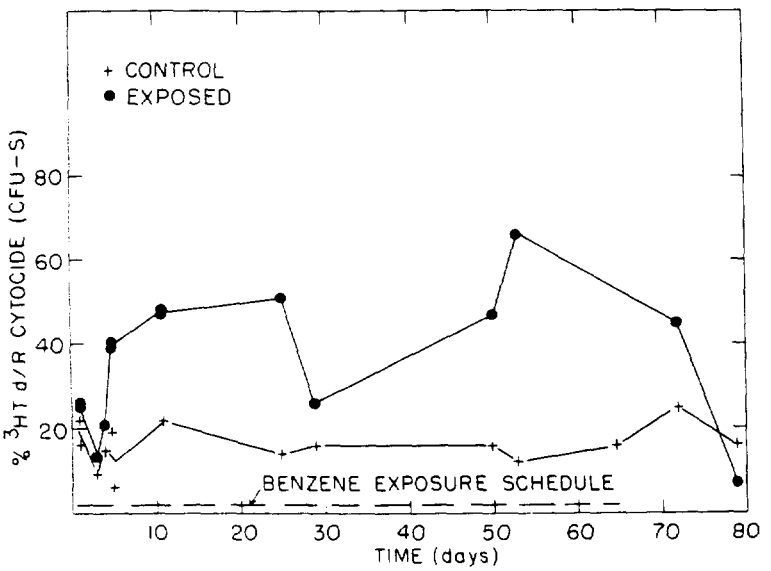

FIGURE 5. Serial studies on [^{3}H]TdR cytocide of CFU-S while mice are exposed to benzene. The cytocide is performed on pooled bone marrow by injecting 6 X 10^4 bone marrow cells incubated with [^{3}H]TdR or cold TdR into each of 25 fatally irradiated mice.

to 48% by d 9 of exposure, and were still elevated on d 25 of exposure. A sharp drop to 26% was observed on d 29 of the study. The highest level (66%) in DNA synthesis was seen on d 53. Seven days after the last exposure, the fraction in DNA synthesis had dropped to 45%, and 14 d after the last exposure it was 7% with total CFU-S still well below control levels (Fig. 4). It appears that the number of CFU-S in DNA synthesis oscillates, but there were not enough determinations to define a cyclic pattern with accurate cycle times and amplitude. In Fig. 6 the serial changes in histological type of splenic colonies produced by bone marrow from benzene-exposed mice is shown graphically. By 3 d of exposure the immature colonies have virtually disappeared. There is an apparent short-lived rebound on d 5 followed by a secondary diminution. The mature erythrocytic and granulocytic colonies diminish more slowly, reaching a minimum on d 5 and remaining at lower than control levels throughout the observation time.

Table 1 shows the projected area of colonies seen in splenic sections. The mean size of erythrocytic spleen colonies in various stages of maturation was smaller when the bone marrow was derived from the benzene-treated mice than when derived from normal bone marrow cells. The smaller size indicates lesser amplification in erythropoiesis. In addition, the small colonies were disintegrating with pyknosis and dispersion of cells to a much greater degree than normal colonies.

The effects of incubating marrow suspensions from benzene-treated

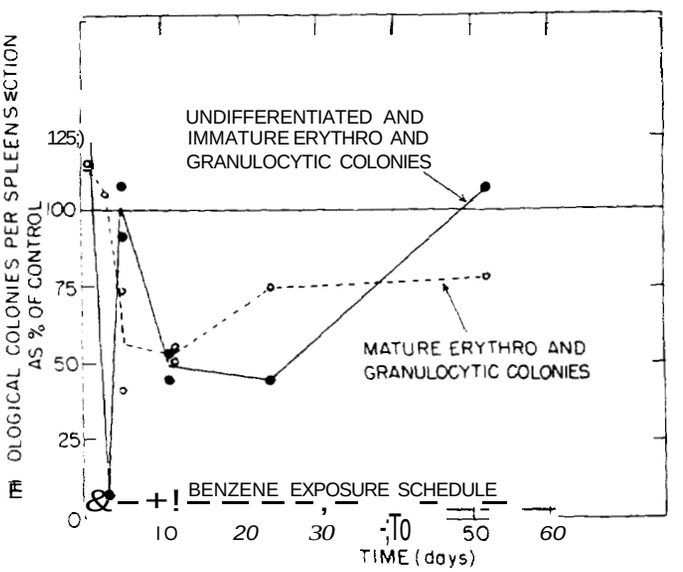

FIGURE 6. Serial studies on histological types of colonies present in spleen sections (H&E stain). Data are expressed as percent of control value at each point. Ten spleens from controls and benzene–xposed mice were studied for each data point.

and control mice with cytocidal concentrations of [^{3}H]TdR are shown in Table 2. Incubation of control bone marrow with [^{3}H]TdR resulted in a reduction of the number of spleen colonies by 28%. When marrow from benzene-treated mice was incubated in the same manner, a 49% reduction in spleen colony number was observed. Thus the fraction of CFU-S killed by [^{3}H]TdR was 28% in controls and 49% in benzene-treated mice. The

TABLE 1. Projected Area of Splenic Colonies' Produced by Bone Marrow Cells from Benzene-treated and Control Mice

Group	Number of spleens	Size of colony in each type[b,c] (mm^2)			
		UNE	ERU	ER	ERM
Control	6		1.71 ± 0.42 (n = 6)	2.32 ± 0.35 (n = 39)	0.75 ± 0.13 (n = 5)
Benzene-exposed	6	1.28 (n = 1)	0.90 ± 0.13 (n = 7)	1.31 ± 0.20 (n = 16)	0.42 (n = 2)

'Histological colonies from middle saggital sections were traced and size was computed by the two diameters measured.

[b]UNE, undifferentiated with trace of erythropoiesis; ERU, erythropoietic immature; ER, erythropoietic all stages of maturation; ERM, erythropoietic mainly nondividing [classification from Reincke et al. (1976)].

[c]Mean ± SE.

TABLE 2 Number of Different Types of Spleen Colonies Seen in Spleen Sections When Bone Marrow Is Derived from Benzene-treated and Control Mice, Each Subjected to [^{3}H]TdR Cytocide

Group		Number[a] of colonies			
		Total	Undifferentiated	ER + GR[b]	ERM + GRM[c]
Control	Cold TdR	101	18	60	20
	[^{3}H]TdR	73	16	52	4
	CFU-S cytocide (%)	28	11	13	80
Benzene-exposed	Cold TdR	71	9	44	16
	[^{3}H]TdR	36	11	22	2
	CFU-S cytocide (%)	49		50	88

[a]The sum of undifferentiated, ER + GR, and ERM + GRM does not equal the total because of the small number of megakaryocytic and unidentifiable colonies observed.

[b]ER, erythropoietic, all stages of maturation; GR, granulopoietic, all stages of maturation.

[c]ERM, erythropoietic, mostly mature and nondividing; GRM, granulopoietic, mostly mature and nondividing.

CFU-S that ultimately produce undifferentiated or highly mature colonies have nearly the same [^{3}H]TdR cytocide. In contrast, 50% of the CFU-S from benzene-treated mice that produce erythrocytic-granulocytic colonies of all degrees of maturation are in DNA synthesis (killed by [^{3}H]TdR), compared to 13% of the CFU-S from control mice.

DISCUSSION

Gill et al. (1980) also reported on the effect of benzene on murine pluripotent stem cells. They found that exposure to 4000 ppm benzene 6 h/d, 5 d/wk, reduced the CFU-S in bone marrow cells from 8.8 to 2.2 per 50,000 cells after 6 wk of such exposure. They did not observe a decrease in bone marrow cellularity and did not calculate the absolute number of CFU-S per femur. Our calculations based on their data show a significant decrease. In our studies, with a different strain of mouse, we observed a striking decrease in absolute marrow cellularity and CFU-S content after 5 d of exposure to 400 ppm benzene for the same exposure schedule. Gill et al. reported that continuous exposure to 500 or 1000 ppm resulted in a decrease in absolute marrow cellularity by 2 d, comparable to the decrease we observed after intermittent exposure. When fatally irradiated mice were transfused with bone marrow cells (5×10^4) and then continually exposed to benzene (100 ppm), 8-d splenic colonies were abolished. These studies with continuous exposure at 100, 500, and 1000 ppm for 4 d showed a progressive decrease in WBCs with time, suggesting that intermittent exposures allow some recovery of the injured precursors of WBCs.

Although Irons et al. (1979) did not study stem cells directly, they did examine the DNA content of the bone marrow cells and [^{3}H]TdR uptake. Their studies showed a significant increase in cells in DNA synthesis and

tetraploid cells and a reduction in the specific activity of [^{3}H]TdR in DNA, indicating an aberration in continuing cell proliferation and the cell cycle. These changes would lead to a reduction in the number of cells produced by the bone marrow. Their studies also indicated that benzene affects the amplification of the stem cell progeny during the presence of benzene and its metabolites. Since there are populations of cells with short transit times in the marrow, these effects do not explain sustained hematopoietic effects after removal of benzene and its metabolites. The cause of long-term effects after benzene exposure must be sought by study of stem cells.

The substantial decrease in absolute marrow cellularity in our studies can only be explained by a large reduction in the amplifying populations of identifiable erythrocytic and granulocytic precursors, since other cell lines account for but a small fraction of the bone marrow cells. The size of these transit populations can be reduced by diminished input from unidentifiable precursor cells (CFU-S and intermediate early progenitors) or by diminished amplification. The elimination of a terminal mitosis would decrease the population of nucleated cells by a factor of 2. A reduction in input from the stem cells by a factor of 2 with constant amplification would also reduce the marrow population within one transit time through the marrow by a factor of **2.** The reduction in absolute number of **CFU-S** in the marrow was greater than a factor of 2. Production of CFU-S was increased, as evidenced by a larger fraction being in DNA synthesis. The number of stem cells produced is directly proportional to the number in DNA synthesis. On d 11, for example, control bone marrow contained 58 X 10^6 cells per leg and marrow from benzene-exposed mice had 20 X 10^6 cells per leg, a reduction by a factor of 3. The CFU-S content is 17.2 X 10^3 per leg in controls and 3.3 X 10^3 in benzene-exposed mice. The number of CFU-S in S phase for controls was 3.61 X 10^3 (17.2 X 10^3 X 0.21) and for benzene-exposed mice was 1.55 X 10^3 (3.3 X 10^3 X 0.47). If the DNA synthesis time is the same in both groups, the production rate of CFU-S in benzene-exposed mice is 1.55 ÷ 3.61, or 0.43 that of the controls. On d 50, marrow from benzene-exposed mice contained 53 X 10^6 cells, compared to **52** X 10^6 cells in controls. The respective CFU-S were 14.1 X 10^3 in controls and 5.8 X 10^3 in benzene-exposed mice with 16 and 47% in DNA synthesis, respectively. Hence, there are 0.16 (14.1 X 10^3) or 2.26 X 10^3 in S phase in controls and 0.47 (5.8 X 10^3) or 2.73 X 10^3 in benzene-treated mice. In order to reconstitute the marrow cellularity by d 50, there must have been a greater production of CFU-S at earlier intervals and/or an increase in the amplification of the transit populations. The problem is of more than trivial interest since others have described hypercellularity during benzene exposure and/or a failure to see a diminution in the marrow cellularity.

In this and other toxicological studies on stem cells in this laboratory, it is evident that the morphology of splenic colonies produced by stem cells from radiation or chemically treated hosts is a useful guide to residual injury. Disintegration, dispersion of cells, pyknosis, and karyorrhexis are seen to a slight degree in colonies produced by stem cells from control mice. The incidence of these is greatly increased when the stem cells are from irradiated and/or chemically treated mice.

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