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Protracted Benzene Exposure Causes a Proliferation of Myeloblasts and/or Promyelocytes in CD-1 Mice

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Exposure to benzene vapor has been associated with pancytopenia and acute myeloblastic leukemia (SNYDER & KOCSIS 1975; GOLDSTEIN 1977). The pancytopenic effects of benzene have been readily reproduced in animals using a variety of treatment regimes (LEONG 1977). On the other hand, the proliferative effects of benzene exposure have been much more difficult to reproduce in animals. Work in our laboratory has resulted in some success in this regard. Protracted exposures of CD-1 mice to 300 ppm benzene vapor produced neutrophilia and 2/40 cases of myeloid leukemia (SNYDER et al. 1978a). Lifetime exposure of C-57BL mice to 300 ppm benzene vapor resulted in 6/40 cases of thymic lymphoma (SNYDER et al. 1980). Both of these hematopoietic neoplasms are exceedingly rare in the respective strains.

It is widely accepted that most leukemias and related disorders are associated with hematopoietic stem cell dyscrasias (QUESENBERRY & LEVITT 1979). Work performed in this laboratory (GREEN et al. in press) and elsewhere (UYEKI et al. 1977; GILL et al. 1980) has shown that benzene exposure causes a reduction in the numbers of pluripotent stem cells (CFU-S) and committed granulocyte-macrophage stem cells (GM-CFU-C). Studies of the effects of benzene exposure on the differentiation and proliferation capabilities of stem cells do not appear to have been reported.

We have recently completed work to assess the effects of protracted benzene exposure on all of the known morphologically identifiable granulocytic cell types. The cells assayed also included the pluripotent stem cell and the granulocyte-macrophage committed stem cell. The purpose of the study was to evaluate which cell(s), if any, within the granulocytic series undergoes preferential proliferation in response to protracted benzene exposure. The exposure regime consisted of 300 ppm exposures for 6 hr/day, 5 days/week for 26 weeks. This regime is similar to one that produced neutrophilia in all exposed CD-1 mice and myeloid leukemia in two mice (SNYDER et al. 1978a).

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METHODS

Housing and Exposures. Male CD-1 mice (Charles River Laboratories, Wilmington, MA), 8-12 weeks old were quarantined for two weeks, acclimatized to a 12 hour light/dark cycle and then randomly distributed into test and control groups. When not in exposure chambers, animals were housed in polycarbonate boxes on wood-chip bedding and allowed food and water ad libitum. Treated animals were exposed to benzene vapor in a 1 m^3 stainless steel and glass dynamic exposure chamber. Air control animals were similarly exposed in a duplicate chamber to filtered, conditioned air. During exposures animals were housed in wire mesh cages and were not provided with food or water. Benzene vapor was generated by passing an air stream over a reservoir of benzene and feeding this benzene-laden air through a heated flask and into the chamber. Chamber concentrations of benzene vapor were monitored six times daily at one hour intervals using an ultraviolet spectrophotometric technique that has been previously described (SNYDER et al. 1978b).

Cell Assays. Three separate 26-week exposures, each with four exposed and four control mice, were performed. Cells from treated or control animals from a particular 26-week exposure were pooled. Organ cellularites and differentials were determined from each of the respective three sets of pooled cells. Stem cell assays were performed on one set of pooled cells from exposed or control animals.

Control and exposed mice were killed by cervical dislocation within three hours of the last exposure. Bone marrow cells were isolated from excised femurs by repeatedly flushing the marrow cavities with ice-cold supplemented McCoys (SMcCoys) medium. Single cell suspensions were prepared by repetitive passage of cells through 23G and 26G needles. Cells were suspended in a known volume of fresh medium after being gently pelleted by contrifugation. Nucleated cell counts were performed on these suspensions using a Coulter ZB-I cell counter. Splenic cells were isolated by pressing the excised spleens through a wire-mesh grid (150 divisions/in^2) and rinsing with 5 mL SMcCoys medium. Single cell suspensions were then prepared as above. Aliquots of these suspensions were used for assays of stem cells and differential cell count.

Femoral and splenic CFU-S were determined by a variant of the method of TILL & MCCULLOCH (1961). Groups of 10 to 15 CD-1 mice were irradiated with 793 rads of 250 kvp X-ray at a rate of about 23.4 rads/min. These mice were then injected via the lateral tail vein with 0.1 mL of cell suspension from either exposed or control mice. Concentrations within the suspensions were adjusted so that the innocula contained 5 x 10^4

bone marrow cells or 5 x 10^5 spleen cells. Recipient mice were housed under sterile conditions for 8-9 days, after which time they were killed and their spleens excised and fixed in Bouins for 24 hours. Macroscopic surface spleen nodules were counted using a dissecting microscope.

The method of BROXMEYER (1978) was used to determine femoral and splenic GM-CFU-C levels. Marrow or splenic cells suspended in SMcCoys were added to sterile test tubes containing 1.0 mL heat inactivated fetal calf serum (HIFCS), 3.0 mL SMcCoys and 5.0 mL 0.66% Difco Agar. Concentrations were adjusted to 5 x 10^4 marrow cells or 10^6 splenic cells per mL. Sterile, Falcon petrie dishes containing 0.1 mL of WEHI-3 as a source of colony stimulating activity were innoculated with 1 mL of cellular suspension. Cultures were plated in triplicate, incubated at 37°C and 5% CO_2 and scored after seven days for the incidence of colonies (> 50 cells) and clusters (3-49 cells).

For differential cell counts, aliquots of marrow or spleen single cell suspensions were concentrated by centrifugation and resuspended in a hypotonic solution consisting of a 3:1 ratio of HIFCS to deionized water. These suspensions were then incubated for ten minutes to allow the cells to swell. Smears were prepared and stained with 1% dimethyoxybenzidine in methanol countered with Wright/Giemsa (LOBUE et al. 1963). A total of 12,000 nucleated cells per organ were scored from treated animals and 9,000 cells per organ were scored from controls. Only cells that could be clearly identified morphologically were categorized. No attempt was made to differentiate the types of blast cells (myeloblast, lymphoblast, erythroblast); they were all counted and categorized as "Blast". Cells not clearly identified were counted and categorized as "Other". The categories of cells used were: Blast, promyelocyte (PMC), myelocyte (MC), metamyelocyte (MM), band (BN), polymorphonuclear cell (PMN), nucleated red cell (NRC), lymphocytic cell (Lymph) and Other.

RESULTS

The integrated mean benzene concentration for the 26 week exposures was 302 ppm.

The percentages of cell-types found in bone marrow are listed in Table 1. With the exception of the polymorphonuclear cells, there was a greater percentage of all cell types in the exposed animals. The numbers of the different marrow cell-types are listed in Table 2. These values were obtained by multiplying the percentages of the cell types by the total number of nucleated cells found in the bone marrow. Since myeloblasts could not be clearly distinguished, these cells are not included in the table. Also listed in Table 2 are

TABLE 1

Percentage of Morphologically Identifiable Cells Catalogued from the Bone Marrow of Exposed and Control Mice

	Exposed	Control
	Mean %[a] ± S.E.	Mean %[a] ± S.E.
Blast	0.86 ± 0.36	0.43 ± 0.19
PMC	3.18 ± 0.78	1.71 ± 0.55
MC	2.37 ± 0.90	0.86 ± 0.44
MM	3.47 ± 0.45	1.87 ± 0.27
BN	5.16 ± 0.60	4.00 ± 0.98
PMN	22.7 ± 1.67	40.3 ± 4.67
NRC	20.4 ± 1.17	13.7 ± 1.56
Lymph	40.5 ± 4.13	35.3 ± 1.84
Other	1.47 ± 0.52	1.00 ± 0.63

[a] Mean of 3 pooled determinations containing a total of 12 animals.

TABLE 2

Number of Cell-Types in the Bone Marrow of Exposed and Control Mice

	Exposed		Control	
	Number	Ratio[a]	Ratio[a]	Number
Nucleated Cells	7×10^6	----	----	21.8×10^6
CFU-S	490	3.6	3.8	6340
GM-CFU-C	1773	126	15.4	24,271
PMC	22.3×10^4	0.8	0.5	37.3×10^4
MC	16.6×10^4	1.5	2.2	18.8×10^4
MM	24.3×10^4	1.5	2.1	40.9×10^4
BN	36.1×10^4	4.4	10.1	87.2×10^4
PMN	158.9×10^4			878.3×10^4

[a] Ratio of the number of progeny cells to the number of cells antecedant: e.g., exposed GM-CFU-C/CFU-S = 1773/490 = 3.6, etc.

ratios of the number of progeny cells to the number of cells antecedant, for example, the ratio of GM-CFU-C to CFU-S, the ratio of promyeloctes to GM-CFU-C, etc. With two exceptions, there is little difference between the ratios in exposed and control groups. There is a major difference between groups in the ratio of promyelocytes to GM-CFU-C. In treated animals this ratio is 126; in controls it is 15.4, an 8-fold difference. There is a less marked difference between groups in the ratio of mature polymorphonuclear cells to band cells. In treated animals the ratio is 4.4 while in controls it is 10.1.

The numbers of blast cells per femur found in exposed and control mice were 6.02×10^4 and 9.37×10^4, respectively. Regardless of lineage, blast cells are believed to be progeny of the pluripotent stem cell (CFU-S). It is appropriate, therefore, to compare the ratios of the numbers of blast cells to the numbers of CFU-S in exposed and control groups. The ratios in exposed and control animals are 123 and 14.8, respectively, an 8-fold difference.

There was an unequal distribution of blasts, promyelocytes and myelocytes among the three groups of pooled spleen cells. There were ten times as many of these cells in one group of pooled cells than in the other two groups (3.7% vs 0.3%). The variable distribution of these cells precluded using these data for any quantitative evaluation of splenic granulocytic cells. The numbers of splenic stem cells were able to be determined, however. In exposed mice, 713 CFU-S and 965 GM-CFU-C were found per spleen. In control mice, 4555 CFU-S and 4789 GM-CFU-C were found per spleen. The ratios of the numbers of GM-CFU-C to CFU-S per spleen in exposed and control mice were 1.4 and 1.1, respectively.

DISCUSSION

There are four dividing cell-types in the granulocytic series. They are, in order of increasing maturity: GM-CFU-C, myeloblast, promyelocyte and myelocyte (CRONKITE 1964). Even though the exposures caused a severe depression in both CFU-S and GM-CFU-C levels, the ratios of GM-CFU-C to CFU-S were almost equal in treated and control mice, indicating no increase in GM-CFU-C proliferation. Likewise, the ratios of myelocytes to promyelocytes were almost equal in treated and control mice indicating no increase in myelocyte proliferation in exposed mice. It is left to conclude that the observed eight-fold increase in promyelocytes in benzene-exposed mice was due to a proliferation of myeloblasts and/or promyelocytes.

The exposures also seem to have produced a proliferation of blast cell-types. This increase could have been caused by an increase in the immediate precursors

to the blasts and/or to a proliferation of the blasts themselves. Although the immediate precursors to erythroblasts (BFU-E and CFU-E) and lymphoblasts (CFU-BL and CFU-TL) were not assayed, the immediate precursor to myeloblasts (GM-CFU-C) was assayed and did not show excessive proliferation in either marrow or spleen.

We have previously reported that prolonged exposure to benzene of several mouse strains produced peripheral blood lymphocytopenia and anemia but also neutrophilia (SNYDER et al. 1978a,b, 1980). It would seem that the proliferation of myeloblasts and/or promyelocytes seen in this study is consistent with the peripheral neutrophilia observed in the previous studies.

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