The Estimation of Toxicity of Benzene and its Metabolites Utilizing Growth of Murine and Human Bone Marrow in Intraperitoneal Diffusion Chambers

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Objectives:

1. Bracket the concentration of benzene in inspired air that produces no detectible suppression and that produces an effect in the growth of murine and human bone marrow growing in plasma clot diffusion chambers (PCDC) and/or liquid diffusion chambers (LDC). PCDC measures the clonal growth and LDC total cell production.

2. Determine whether benzene and its metabolites act at the pluripotent stem cell, early, cytologically, nonrecognizable progenitors (BFU-E 3 and 8 day, CFU-E 2 day, CFU-GM or bipotent precursors), and/or cytologically recognizable amplifying transit cell populations of murine and human bone marrow.

Background:

Benzene inhalation has produced hypoplasia to aplasia of the bone marrow in man. The time of exposure and concentration of benzene are not well-established (1,4). A significant fraction of human beings that survived marrow hypoplasia developed acute leukemia (myeloblastic, erythroblastic, and lymphoblastic) (1,3). Synder et al. (4) have shown that exposure to benzene 300 ppm 6 hrs/day, 5 days/week for life induces a small increase in lymphoma in C57Bl/6J mice. We have shown that exposure to 300 ppm 6 hrs/day, 5 days/week for 16 weeks induces a higher incidence of leukemia and solid tumors occurring in two waves. The present total incidence of neoplastic disease is about 25% compared to 1% in the nonexposed comparison C57Bl/6J BNL female mice. The study is still under way. At this point one can conclude that benzene is leukemogenic with mouse and that benzene plus other ill-defined exposures combined were leukemogenic in man.

The hematopoietic effects in mice are being studied by several groups (4-9). Exposure to 100 ppm benzene or more for 6 hrs/day for 5 times will produce a lymphopenia, modest anemia, decrease in marrow concentration of pluripotent stem cells (spleen colony-forming units--CFU-S), BFU-E 3 and 8 day, CFU-E, GM-CFU-C reduction in total bone marrrow cellularity and an increase in the fraction of CFU-S that are in DNA synthesis.

The assay for CFU-S involves injection of bone marrow from treated and normal mice into fatally irradiated mice which are killed 7-10 days later, spleens removed, and the number of macro colonies counted. The results can be expressed as a fraction of the control mice or as absolute number per femur (10).

The assays for burst-forming unit erythroid (BFU-E), colony-forming unit erythroid (CFU-E), granulocytes, macrophage colony-forming unit (GM-CFU-C), and granulocyte, erythroid, megakaryocyte, and macrophage (GEMM) are in vitro cultures (11-13). The cytologically unidentifiable

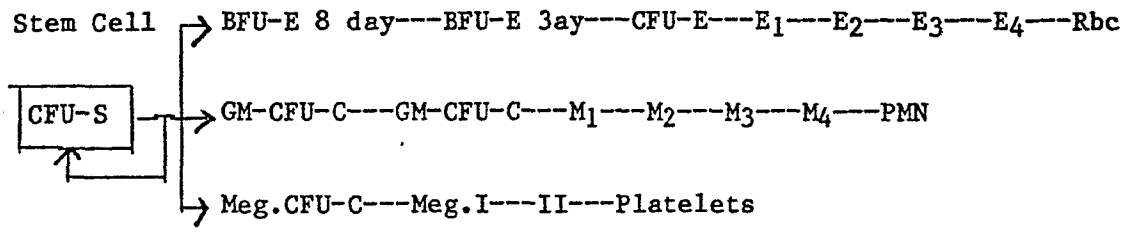
precursors have receptors for specific humoral regulators such as erythropoietin (Ep) burst-promoting activity (BPA), colony stimulation factor (CSF), stem-cell stimulator, etc. The in vitro requirements are the presence of precursor cells with appropriate formed and functioning receptors and the required stimulatory molecules. For each stimulating regulatory molecule there appears to be an inhibitor. The in vitro assays can be used to test for specific stimulatory or inhibitory molecules.

Diffusion chamber culture takes two forms: the liquid diffusion culture (LDC) and the plasma clot diffusion culture (PCDC). The history on development and use of these cultures is presented in detail in Cronkite and Carsten's book (14). The diffusion chambers consist of a lucite ring with a small hole. Millipore or Nuclepore filters (0.2 uM pore) are heat-sealed on the ring. A suspension of target cells is injected into the chamber and the hole is closed with a nylon plug. Two chambers are placed in the peritoneal cavity of the mouse. The mouse provides the humoral regulatory molecules for proliferation and differentiation down the different hemopoietic pathways. The host mouse can be treated in different ways to increase or decrease the flux of regulatory molecules into the chamber and thus alter the rate of proliferation and/or differentiation. Hypoxia increases the production of Ep and other factor(s) that accelerate erythropoiesis (1). Sterile inflammation increases the flux of CSF and other molecules increasing rate of granulopoiesis. Transfusion plethora decreases Ep and erythropoiesis.

Does mouse metabolize benzene as man

If one administers drugs or toxic substances to the host mouse by any route (inhalation, ingestion, or injection), the material can be processed by hepatic and other enzymes (cytochrome P 450-dependent hydroxyl radical-mediated reactions, by mixed-function oxidases, etc.) and the metabolites will be able to enter the diffusion chamber. The amount entering will be a function of their half-life and transit time to the LDC or PCDC. Thus one can study the effect of benzene and its metabolites on proliferation and differentiation of hemopoietic precursor cells of man, mouse, or other mammal implanted in the LDC or the PCDC.

Hematopoiesis consists of:



Self-renewal

-----Progressive growth of receptors and maturity----->
-----Exponential amplification depending on humoral stimulating regulatory molecules----------->
Stem Cell -----10-12 mitoses depending on humoral stimulating regulatory molecules 1024 to 2047---------->Rbc

Thus, toxic substances entering the diffusion chamber may act in one or more ways:

1. Inactivate host regulatory molecules

2. Inactivate receptors on the target cells

3. Attack and inactivate genes responsible for control of receptor synthesis in target cells.

4. Kill target cells

Inactivation of host regulatory molecules can be tested by assaying the stimulatory effect of the blood serum of benzene-treated animals to serums of comparison nontreated mice on the target cells of bone marrow.

Inactivation of receptors on target cells can be tested by using radiolabeled regulatory molecules and see if the uptake on target cells of benzene-treated mice is reduced using the autoradiographic technique of Shadduck et al. (16).

The inactivation of genes responsible for synthesis of receptor proteins is more difficult to determine. This would require separation and concentration of stem cells (CFU-S) and their growth in LDC and/or PCDC to see if they can produce BFU-E, GM-CFU-C when the host is given the appropriate stimulus (hypoxia, inflammation, etc.).

The killing of target cells is approached by quantitative assay of their number in the appropriate assay system although killing outright is difficult to distinguish from failure to respond to the appropriate stimuli.

The techniques for diffusion chamber culture of murine CFU-S, BFU-E, GMCFU-C, total and differential are established. The techniques for culturing of human bone marrow in LDC and PCDC for assay of GM-CFU-C, total and differential cellularity are established. Techniques for culture of human erythrocytic, megakaryocytic, and multipotential cells in PCDC are not well-established. An in vitro technique for culture of a multipotential human bone marrow early progenitor is established. This could be applied to culture of human bone marrow cells growing in diffusion chambers but as yet, to my knowledge, has not been attempted.

Experimental Design:

The proposed research only addresses the effect of benzene upon hemopoietic cell proliferation and differentiation. Research towards mechanism of action at cellular or genetic level is not included.

C57Bl/6J BNL mice will be used as the recipients of LDC and PCDC containing murine or human bone marrow. Each mouse will have either two LDC or two PCDC. Equal numbers of mice will be exposed to benzene or submitted to sham exposure. The following concentrations of benzene in air will be used: 0, 1, 10, 100, 200 and 400 ppm. The mice will be exposed 6 hrs/day, 5 days/week.

The recipient mice in whom erythropopiesis is studied will be exposed to hypoxia (18,000 ft.) for 6 hrs in addition to the 6 hrs. exposure to benzene. The recipient mice in whom proliferation of CFU-S and granulocytic precursors is studied are given 650 rads whole body radiation on the day and before the diffusion chambers are implanted. Nonirradiated

and nonhypoxic mice are used for comparison. Hypoxia on day 1 and 2 produces extra amounts of Ep and other stimulatory molecules to initiate clonal growth (15). Radiation of hosts provides humoral stimulation of the CFU-S and early GM-progenitors.

The protocols for study of the effect of benzene on the different aspects of hematopoiesis follow.

CFU-S and granulopoiesis will be studied together. Nonirradiated and 650 rads-irradiated host mice will be used. Each host mouse will have two diffusion chambers implanted in the peritoneum. From our previous work (Boyum et al.,(17)) chambers will be harvested at 0, 1, 2, 4, 6, 8 and 10 days for total cellularity. For study of CFU-S proliferation, chambers will be harvested on days 0, 1, 2, 3 and 4. To study the effect on CFU-S, 10^5 bone marrow cells will be put in LDC, and to study total production of cells in LDC and clonal growth in PCDC $2x10^4$ murine bone marrow cells will be used.

For the CFU-S assays the cells in the 12 LDC in 6 mice will be pooled, counted, and divided into 15 equal amounts for intravenous injection into 15 lethally irradiated mice. These will be killed on day 9, the spleens fixed in Bouin's for 24 hrs., and the spleen colonies counted.

The LDC for total cellularity are also counted and then pooled. Smears are made for differential counts.

The PCDC are fixed in 5% formalin, washed, stained with hematoxylin eosin, cleared, and the clots mounted on glass slides and the number of colonies counted, each being a clone.

The means and standard errors will be calculated. Graphs will be plotted showing changes in the number of the different items measured versus time in diffusion chamber. A family of curves will be obtained showing the effect of inhalation of the different concentrations of benzene upon the end points measured. Point by point will be tested for significance in difference of the means at each time point and concentration of benzene.

Experiment II:

In this study we will compare the effect of benzene simultaneously on the growth of murine and human cells in diffusion chambers in the same mouse. One LDC or PCDC will contain murine cells, the other will contain human cells. On days 0, 3, 5, 7, 10, 14 and 17 LDC will be harvested, the human cells and murine cells will be pooled separately, counted, smears made and stained. The pooled cells will be divided into 6 aliquots but not more than $5x10^4$ cells will be plated in agar for in vitro assay of the number of GM CFU-C present.

The PCDC will have $2x10^4$ human or murine bone marrow cells and will be harvested on day 0, 5, 7, 14 and 17. They will be stained with esterases positive for macrophages (black) and granulocytes (red).

In both parts of this study, the LDC and PCDC are reimplanted in new mice on the 7th day. If this is not done, host (particularly the irradiated) mice may die. As mentioned earlier, murine growth peaks around day 7 or 8 and with human marrow around day 14.

Data will be treated as in Experiment I.

Experiment III will involve study of the effect of benzene on erythropoiesis in PCDC. As discussed earlier, the technique for growing human erythrocytic progenitors in PCDC has not been established. We would first propose a pilot study to see if human erythrocytic progenitors respond to the same stimuli as murine erythrocytic progenitors as follows.

Place 10^5 human bone marrow cells in 12 PCDC without hypoxia and 12 PCDC with hypoxia. Harvest 6 PCDC on day 7 and 14 and observe for erythrocytic bursts. If bursts are found, then proceed with a direct comparison of the effect of benzene upon murine and human erythropoiesis as in Experiment II where each mouse will have a PCDC with human cells and a PCDC with murine cells. The PCDC will be fixed, stained, and the colonies counted.

The data will be treated as before.

Data Analysis:

From the results of exposure to 0, 1, 10, 200 and 400 ppm of benzene, one will be able to construct a family of curves as a function of time in culture and concentration of benzene. I added the 200 ppm because it is my considered opinion that no significant effect will be detected at 10 and 10 ppm, leaving a probable effect at 100 and 400 ppm. From two data points one cannot tell whether the response is linear or curvilinear--the latter perhaps more likely. It is recommended that the intermediate concentrations of 200 ppm benzene be retained. If this is not desired, the cost for mice and diffusion chambers would be decreased by 1/6 (17%).

All data will be entered into our computer for the appropriate analysis for significance of the difference between means. The means will be plotted as a function of time in culture and concentration of benzene inhaled. Regression lines, if appropriate, will be calculated.

If there is a comparable response of human and murine marrow where similar end points are available, it would imply that the mouse is a reasonable surrogate for the response of human marrow to the metabolites of benzene that are formed in the mouse and enter the LDC or PCDC.

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Experiment I - Study of Effects of Inhalation of Benzene on Stem Cells (CFU-S) and Granulocytic Macrophage Progenitors of Mice

	Number of Mice		
	Liquid Diffusion Chamber		PCDC for GM-CFU-C
Days	CFU-S	Total Cellularity	
0	6	6	6
1	6	6	
2	6	6	
3	6		
4	6	6	6
6			
18		6	6
10		6	
Total	30	36	18

Subtotal	84	
For six benzene doses x 6	504	
Control and irradiated hosts x 2	1008	1008
Mice for assay of CFU-S each time point 15 x 5	75	
Control and irradiated hosts x 2	150	
For 6 benzene doses x 6	900	900
Total mice		1908

Experiment II - A Comparison of the Effect of Inhaling Benzene upon Hemopoietic Growth of Murine and Human Bone Marrow in the Same Host Mouse

Number of Mice

Days	LDC	PCDC
	Total Cellularity, Differential, & GM-CFU-C	
0	12	12
3	12	
5	12	12
7	12	12
10	12	
14	12	12
17	12	12
	84	60
	Reimplantation 36	24
	120	84
		Subtotal 204
	For normal and irradiated x 2	408
	For 6 benzene doses x 6	2448
	Diffusion Chambers 204 x 2	408
	Normal and irradiated hosts x 2	816
	For 6 benzene doses x 6	4896

Experiment III - Effect of Inhalation of Benzene upon Proliferation of Erythropoietic Progenitors of Man and Mouse in PCDC

Number of Mice

Days	Normoxic	Hypoxic
0	12	12
2	12	12
6	12	12
8	12	12
10	12	12
12	12	12
14	12	12
	84	84

168 Mice
336 DC

For 6 concentrations of benzene 6 x 168 1008 mice

For 6 concentrations of benzene 6 x 336 2016 DC

** If the human cells will not grow in the PCDC, the number of mice and the cost of this phase will drop by about 56%.