

FINAL REPORT
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ENVIRONMENTALLY-INDUCED MALIGNANCIES: AN IN VIVO MODEL TO
EVALUATE THE HEALTH IMPACT OF CHEMICALS IN MIXED WASTE

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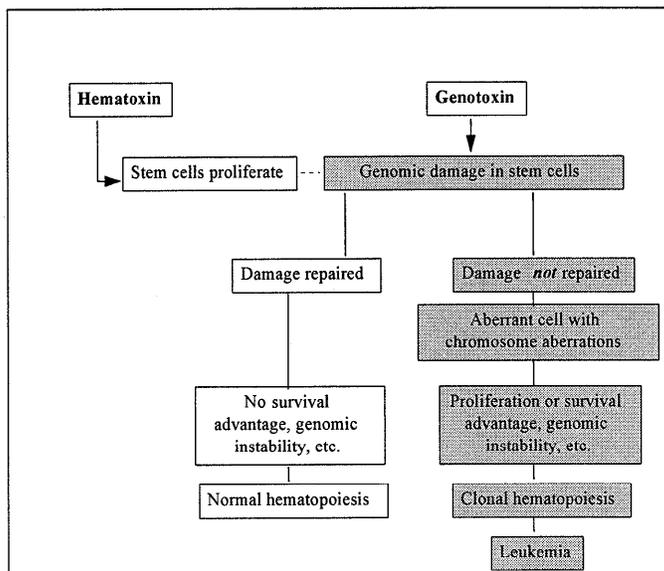
Work Scope and Research Objectives

Occupational and environmental exposure to organic ligands, solvents, fuel hydrocarbons, and polychlorinated biphenyls are linked with increased risk of hematologic malignancies. DOE facilities and waste sites in the U.S are contaminated with mixtures of potentially hazardous chemicals such as metals, organic ligands, solvents, fuel hydrocarbons, polychlorinated biphenyls and radioactive isotopes (1). Fuel hydrocarbons (e.g., benzene, toluene, xylene and ethylbenzene) are the most commonly reported class of compounds found in DOE sites occurring in 65% of the sediment and 55% of the ground water specimens obtained at DOE facilities and in ~ 25% of Waste Sites. Radionuclides are present in groundwater/sediment in > 40% DOE facilities and Waste sites. Epidemiological studies have established that exposure to fuel hydrocarbons is associated with an increased risk of acute myelogenous leukemia in humans (2-5). Benzene (bz) and trichlorethylene (tce) exposure are also associated with development of non-Hodgkin's lymphoma, multiple myeloma, and other hematopoietic malignancies (4,6,7). Ionizing radiation increases leukemia risk in both humans (8) and animals (9,10,11). Mechanisms underlying progression to malignancy following radiation and chemical exposures are unknown. Importantly, the relationship between genotoxin exposure and carcinogenesis is poorly understood. A major goal of this project was to establish linkage between chemical/radiation exposure and induction of genomic damage in target populations with the capability to undergo transformation.

Carcinogenesis is characterized by progressive accumulation of genomic aberrations. Exposure to environmental genotoxins may increase the number of genomic aberrations if the DNA errors are unrepaired or misrepaired. DNA damage incurred by cells/subpopulations with long life spans is more likely to increase susceptibility to leukemic progression than DNA damage in cells with limited life spans (i.e., differentiated cells). Cells with long life spans and proliferative capability have more opportunities to incur multiple genetic hits, which if unrepaired can be passed to daughter cells. Hematopoietic stem cells (hsc) persist for long periods (i.e., years) and maintain hematopoiesis by hierarchical amplification of progeny in multiple lineages. Thus, long-lived hsc with damaged genomes may produce genetically aberrant progeny in multiple lineages. Genotoxic effects of radiation and chemical exposures on the genomes of hsc subpopulations are unknown and the importance of accumulated genomic aberrations in this population for leukemic progression is not established. Hsc from patients with myeloid leukemia and myelodysplasia carry similar genomic aberrations as the leukemic clone (12-14). Hsc from leukemic patients also show dysregulated cell cycle regulatory protein expression (15), suggesting existence of cell cycle abnormalities in primitive cells. These observations suggest that genomic and functional abnormalities in hsc may precede and/or contribute to the leukemic clone, and indicate the importance of understanding radiation and chemical effects on this subpopulation. Some chromosomal aberrations are associated with induction of genomic instability in cell lines *in vitro* (16). These concepts were integrated into a model of environmentally induced leukemogenesis (Figure 1) in which unrepaired DNA damage in hsc leads to genetically aberrant hsc subpopulations and eventually to

development of genomic instability, which provides a survival advantage for clonal populations. We postulated that exposure to radiation and chemicals with leukemogenic potential would induce genomic aberrations in hsc and downstream progeny. Furthermore, since most tumor cells are believed to be genomically unstable (hence the high rate of genomic aberrations), we postulated that agents associated with leukemogenesis would

Figure 1. Model of Leukemogenesis



induce genomic instability, reflected in cell cycle checkpoint inactivation. Several aspects of this model were evaluated during the course of our studies.

The goals of this project were to identify, quantify and characterize radiation and chemical induced genomic aberrations in hsc to lend insight into the early events involved in radiation and chemicals associated hematologic malignancies. Bz, tce and radiation increase risk of myeloid leukemia.

Radiation induces stable, chromosomal translocations (TL). Bz and its metabolites increase the frequency of aneuploid cells or lymphocytes *in vitro* (17,18), and in some studies cause point mutations, partial chromosome deletions and translocations (19,20), albeit at a much lower frequency than aneusomies. Bz-exposed individuals carry more aneuploid lymphocytes than controls (20-23). Tce is a weak genotoxin that induces micronuclei, DNA adducts, and aneusomies in blood lymphocytes (24,25). The experimental design to evaluate radiation, bz and tce effects on hsc and downstream progeny included studies to: a) demonstrate that radiation and chemicals induce genomic aberrations in hsc, b) evaluate the role of cell cycle status in influencing the amount and persistence of genetically damaged hsc, c) establish whether bz and tce inactivate cell cycle checkpoints which control genomic stability, and d) to evaluate the leukemogenic potential of low dose radiation and bz exposures in mice. We anticipated that these studies would increase understanding of steps in the progression pathway of chemically induced leukemia, eventually contribute to development of strategies to evaluate the leukemogenic potential of environmental contaminants and improve biomonitoring and risk assessment.

Specifically, our objectives were to use a mouse model to

1. Determine whether radiation and chemicals induce genomic damage in hcs and whether recruitment of hsc into cycle prior to genotoxin exposure increases genomic damage.
2. Determine whether environmental genotoxins with leukemogenic potential disrupt hsc genomic integrity by inactivating cell cycle checkpoints.

3. Test the hypothesis that chronic low dose exposure to radiation, benzene and TCE will result induce continual hsc cycling, increase the frequency of genetically aberrant hsc and elevate genomic instability as compared to the same dose administered acutely.

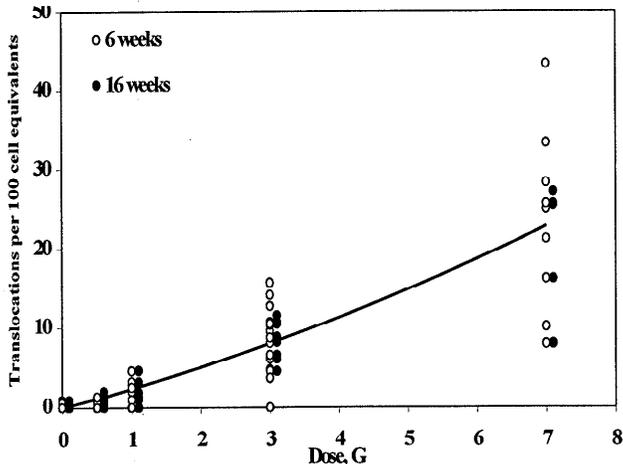
Methods and Results

Molecular and cellular approaches were used to test components of the proposed model of leukemogenesis and to establish the relationship between chemical/radiation induced genomic aberrations, cell cycle status at time of exposure, and genomic instability in hsc and downstream progeny. Unrepaired and misrepaired DNA damage in lymphoid, myeloid and hsc populations from irradiated mice were quantified using fluorescence in situ hybridization (FISH) (26) with DNA probes specific for mouse chromosomes X, 1, 2, 3, and 11 (27,28). Unrepaired damage arising from bz and tce exposure was quantified by enumerating aneusomies in isolated hemopoietic subpopulations. Selection of DNA probes to quantify persistent DNA damage was based upon availability, as well as synteny with regions of the human genome frequently involved in aberrations in hematologic malignancies (29). FISH was performed essentially as described by Pinkel *et al.*, (26) with modifications for dual-color detection of fluorescencated hybrids. Chromosomal aberrations were quantified in circulating mature lymphoid and myeloid cells, and in hsc isolated from bone marrow using multivariate flow cytometric analyses and sorting (30,31). Hemotoxicity was measured using colony forming assays that detect progenitors capable of contributing to the myeloid/granulocyte lineage (CFU-GM) and long-term culture assays (cobblestone area forming cells; CAFC) (32) that probe a spectrum of immature and primitive hemopoietic subpopulations, including hsc capable of generating multilineage hematopoiesis. Cell cycle checkpoint assays were performed as described previously (16,33). Additional discussion of these techniques accompanies the data/results below. Data and results are presented in three sections: radiation and chemical induced genomic aberrations in hsc, genomic instability in bz treated cells, and mouse models of leukemia.

Radiation and chemical induction of genomic aberrations in hsc and downstream progeny

Radiation/chemical genotoxicity, cytotoxicity and exposure dose are frequently estimated using reporter populations in blood, which is a readily accessible tissue. The translocation frequency response (tfr) measured using peripheral blood lymphocytes is widely used for radiation biomonitoring and dose estimation (34-37). Longitudinal studies on sequential specimens collected from patients after the Estonia accident suggest that the TL frequency remains constant for ~2 years (37). Biannual blood sampling of Chernobyl victims indicated that the TL frequencies 5-8 years after exposure are constant (35). The persistence of lymphocytes with genomic damage more than four decades after exposure suggests that a subset of lymphocytes (i.e. 10-25%) may be long lived or that aberrant blood lymphocytes continue to be produced from genetically-damaged precursor cells in other tissues (38). It is unknown, however, whether genomic aberrations measured in reporter populations reflects damage in cells at risk of transformation (i.e., hsc). We postulated that radiation induces aberrations in hsc and that these aberrations will persist in hematopoietic tissues for extended periods. Furthermore, we hypothesized that

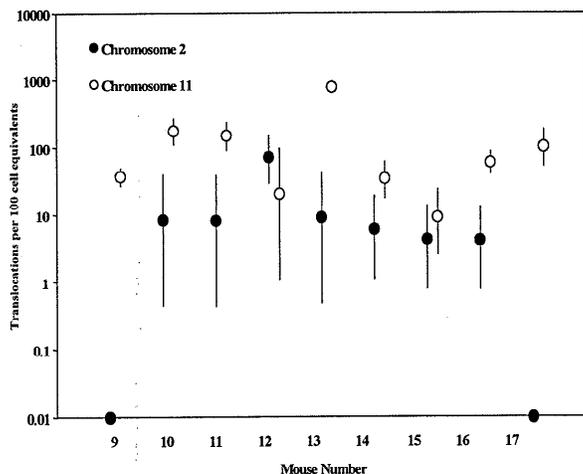
Figure 2. TL frequency dose response in C57Bl lymphocytes 6 and 16 weeks after irradiation *in vivo*.



Measurements for chromosomes 1+2 and X+3 are combined into a single dataset. Data points for the 6 and 16 week measurements are slightly offset for clarity. The dose-response curve was fit by modeling the pooled data using a linear-quadratic formula, $y = 2.24D + 0.145D^2$, where y is the number of TLs per 100 cell equivalents (CE) and D is the dose in Gy. Each data point represents the TL frequency in lymphocytes from an individual mouse.

weeks after exposure, respectively. A linear quadratic dose-response similar to those seen by other authors (39) was defined. The sensitivity of the tfr assay is limited to doses

Figure 3. Chromosome TL 20 months after 3 Gy



Data are shown for 9 irradiated C57Bl mice. Bars represent the 95% confidence interval for each measurement. Mouse 9 and 17 did not carry chromosome 2 TL. For clarity, these two data points are plotted at 0.01/100CE.

pronounced at the 7Gy dose. These data demonstrate that TLs in circulating lymphocytes are relatively stable for at least 4 months post exposure.

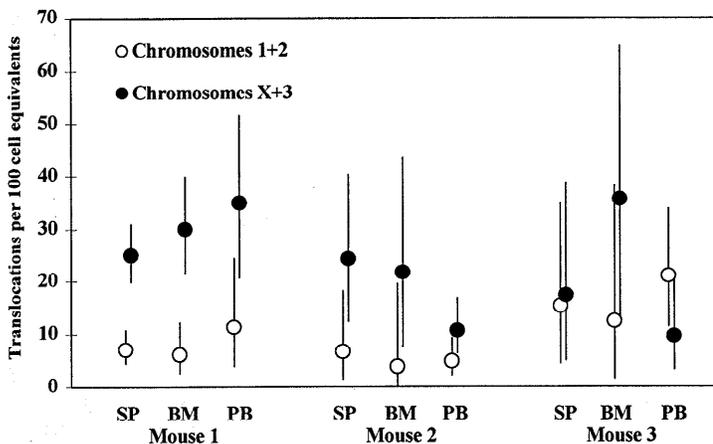
exposure to DNA damaging agents that induce proliferation of the normally quiescent stem cell population would increase the frequency and persistence of genomic aberrations in hsc and generate higher numbers of hsc.

We first established the dose-relationship of lymphocyte TLs in C57Bl mice at 6 and 16 weeks after whole body doses of 0, 0.5, 1, 3 and 7 Gy, using FISH probes for chromosome pairs 1+2 and X+3. The TL frequency is independent of the chromosome used and constant from 6-16 weeks after exposure (Figure 2). The average TL frequencies in peripheral blood lymphocytes from mice irradiated with 1, 3 and 7 Gy are $1.6 \pm 1.0\%$, $9.2 \pm 2.1\%$ and $20.6 \pm 8.8\%$, respectively, 6

of ≥ 1 Gy because the TL frequency (0.4 TL/100 CE) at 0.5 Gy is similar to the spontaneous TL frequency in unirradiated mice. Sensitivity could be improved by increasing the number of metaphases scored (40). The tfr after 1 and 3 Gy is similar among mice, but variation is more

We extended the analyses to determine the persistence of genetically aberrant lymphocytes during lifetime hematopoiesis. We determined that all irradiated (3 Gy) mice carried genetically aberrant circulating lymphocytes for at least 20 months after exposure (Figure 3). The lifespan of C57Bl mice approximates 24-26 months. Interestingly, we also determined that chromosome 11 TLs in blood lymphocytes are ~

Figure 4. Radiation induced TLs in multiple hemopoietic tissues 1.5 years after exposure



Data are shown for splenic (SP), bone marrow (BM) and peripheral blood (PB) lymphocytes from irradiated C57Bl mice. Bars represent the 95% confidence interval for each measurement.

10-fold ($58.7 \pm 20\%$) more prevalent than chromosome 2 TLs ($5.4 \pm 19\%$) ($p < 0.01$) (Figure 3). The frequency of chromosomes 11 and 2 TLs vary substantially among mice. Together these data suggest that 1) quantitation of the tfr using FISH with chromosome-specific DNA probes is influenced by the chromosome selected for measurements, 2) genetically aberrant lymphocytes persist

for the lifetime of the animal and 3) chromosome 11 TLs may provide a survival advantage for lymphocyte subpopulations.

The tfr assay assumes that genetically aberrant lymphocytes in the circulation at long times after exposure derive from genetically aberrant hsc or lymphoid precursors. Numerous studies report elevated TL frequencies in lymphocytes many years after exposure (i.e., 2-40 years) (41-43). The half-life of human lymphocytes is unknown, although estimates range from 6 months to 6 years (38). If hsc with damaged genomes continually generate genetically aberrant lymphocytes, we predicted that lymphocytes with TLs should be present in multiple hematopoietic tissues. The frequency of aberrant lymphocytes in blood, spleen and marrow with chromosomes 1+2 and X+3 TLs 1.5 years after exposure was measured (Figure 4). TL frequencies in all tissues range from 5-35 TL/100 CE. TL for chromosomes X+3 are more frequent, on average, than chromosomes 1+2 (means $21.9 \pm 9.5\%$ for X+3 and $8.0 \pm 5.7\%$ for 1+2, $p < 0.01$). However, once chromosome-specific preferences are considered, the frequency of aberrant lymphocytes in the circulation, spleen and marrow are similar within the same animal. These data are consistent with derivation of cytogenetically aberrant lymphocytes from a radiation damaged lymphoid/stem precursor. Complete details of this study can be found in Giver et al (44).

In order to confirm that radiation induces genomic aberrations in hsc, and that hsc with damaged genomes persist for long time periods, TL were quantified in hsc isolated from mice irradiated 1.5 years prior to tissue collection. A hsc subpopulation (c-kit⁺sca-1⁺lin⁻) was discriminated using flow cytometry. Hsc subpopulations were sorted, cultured in a

Table 1. TL in Hsc in Aged, Irradiated mice

Group	No. Metaphases	Translocations			
		2 (%)	CI	11 (%)	CI
Control	1410	0	0-0.2	0	0-0.2
3 Gy	2062	3 ^b	2.2-6.4	9.2	7.9 - 10.6
FU	1462	0 ^a	0-0.2	0	0-0.2 ^a
FU-2d-3Gy	3173	0.7 ^{ab}	0.4-2.0	2.3	1.8-2.8 ^a
FU-4d-3Gy	3740	0.1 ^a	0-0.2	0.2	0-0.3 ^a

CI: confidence interval

^a p <0.001 compared to radiation alone

^b p <0.001 compared to chromosome 11

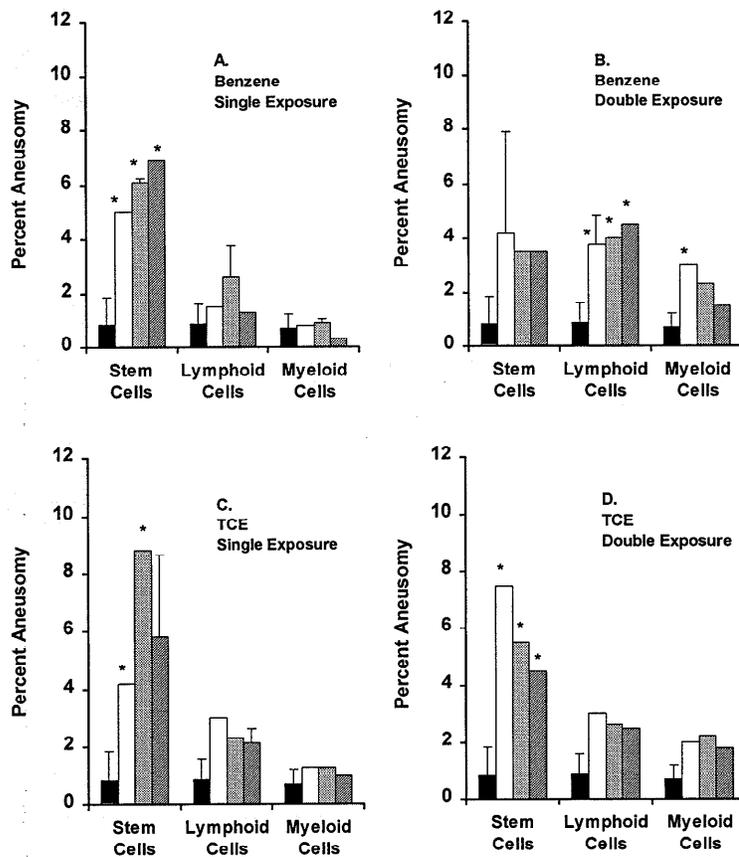
liquid suspension, and metaphases harvested after colcemid block. TLs involving chromosomes 2 and 11 were quantified (Table 1). Genetically aberrant hsc were not detected in unirradiated, age-matched mice. However, the TL frequency approximated 12%.

in hsc subpopulations from irradiated mice. Chromosome 11 TLs were more prevalent than chromosome 2 TLs. Thus, genetically damaged hsc persist during lifetime hematopoiesis. Furthermore, it is likely that the high prevalence of circulating lymphocytes with chromosome 11 TL reflects propagation from genetically aberrant hsc with the same TLs. Genomic aberrations that impact pathways involved in DNA repair, apoptosis, etc. may represent the first “genetic hit” in a multistep process. In these cases, subsequent exposure to the same or other genotoxins will contribute to accumulation of additional genomic errors in both hsc and maturing populations. One of the aims of this project was to test the hypothesis that the cycling status of hsc at the time of genotoxin exposure impacts the amount and persistence of genomic damage. We postulated that cycling hsc would accumulate more genomic aberrations than quiescent hsc and that these changes would result in a higher frequency of genetically aberrant clonal progeny. We tested this hypothesis by recruiting hsc into cycle prior to radiation exposure and then quantified genomic aberrations in hsc subpopulations present 1.5 years after irradiation. 5-Fluorouracil (FU) is an S-phase specific agent that recruits hsc into cycle. Recruitment of hsc into S-phase is highest at 4 days post FU, and somewhat lower at 2 days. Thus, we expected to observe a higher frequency of hsc with damaged genomes in mice irradiated 4 days after FU than after 2 days and compared to irradiated mice that did not receive FU. However, our data do not confirm this hypothesis. Mice irradiated 4 days after FU showed fewer cells with aberrations than mice that received radiation alone 2 days after FU (Table 1). Thus, these data indicate that recruitment of hsc into cycle reduces, rather than increases, the frequency of genetically damaged cells. Reduced numbers of aberrant cells may be due to commitment of hsc to differentiation pathways in order to replace cells killed by the combined radiation/FU treatment.

The findings from the cycling studies have implications for exposure to agents that are both genotoxic and hematotoxic (i.e., benzene). It is generally assumed that increasing radiation or chemical exposure doses are associated with increased adverse health effects. Indeed, our data indicate more genomic damage with 3 Gy than 0.5 Gy, although, as discussed below, relationships between chemical dose and genomic damage are less clear. The cycling studies suggest that simultaneous exposure to agents that are hematotoxic (i., 5-FU, radiation) and genotoxic (radiation) will result in less genomic damage than to either agent alone in hsc, presumably due to high demands for rapid differentiation placed on the hsc population.

Genomic aberrations in hsc following bz and tce exposure

Figure 5. Aneuploid hsc after bz and tce exposure *in vivo*

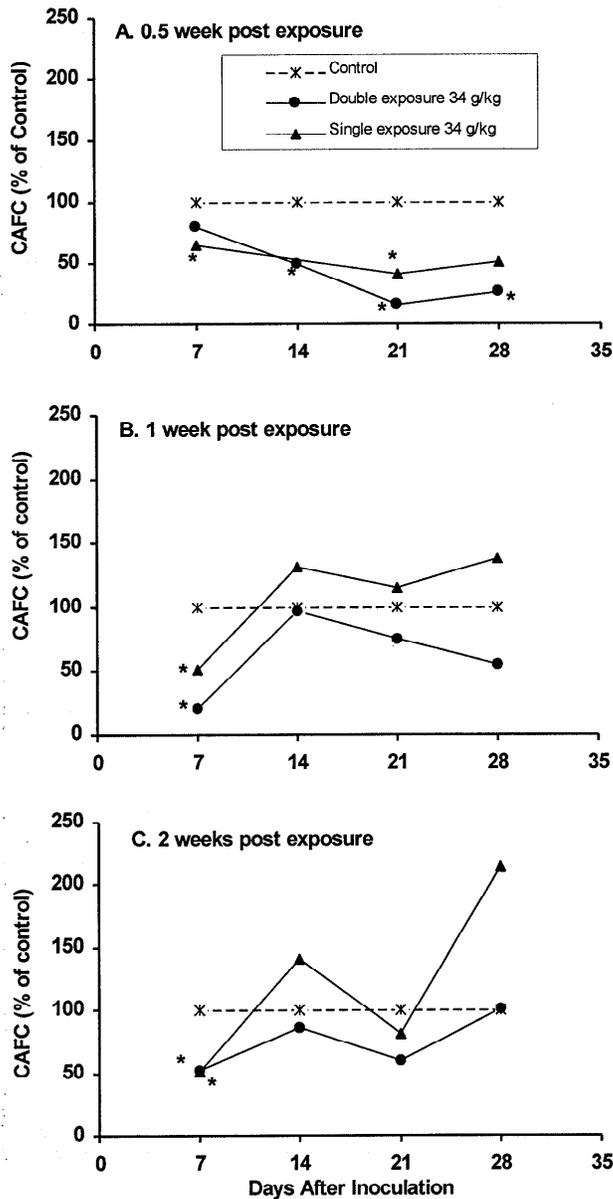


Aneuploid cells quantified 6 days after dermal benzene or TCE exposure. For benzene exposures; black; control, white; 8.5 g/kg, light gray; 17 g/kg, dark gray; 25.5 g/kg. A) bz, single exposure; B) bz, two exposures, 4 days apart. For tce exposures; black; control, white; 7 g/kg, light gray; 14 g/kg, dark gray; 28 g/kg. C) tce, single exposure; D) tce, two exposures, 4 days apart. * indicates values significantly different than control, $p > 0.05$.

Genomic aberrations in sorted hsc, circulating lymphocytes and marrow myeloid cells were measured six days after single or repeated exposures to bz or tce. Aneusomies represent the predominant aberration observed; translocations and deletions were rare. The majority (80%) aneuploid cells from control and chemically treated mice display loss of a single copy of either chromosome 2 or 11. Aneuploid cells from control mice consistently comprise <1% of less of each subpopulation. In contrast, hsc subpopulations from mice that received a single topical bz exposure (bz; 8.5, 17 or 25.5 g/kg), contain approximately 5, 6 and 7% aneuploid cells, respectively, significantly elevated above control values (Figure 5a). Aneuploid hsc from mice exposed to two benzene

doses were also elevated: however, the increase was not significant (Figure 5b). Aneuploid lymphoid and myeloid cells approximated controls after a single bz dose, however, the aneuploid lymphocytes exceeded control values after two bz exposures. The increase in aneuploid cells after bz exposure appears to be dose-independent. The

Figure 6. Bz and tce toxicity to hematopoietic progenitors and hsc



Cobblestone area forming cell (CAFC) frequencies from bz treated mice. Frequencies are shown as percentage CAFC per femur compared to control values. * Significantly different than controls ($p < 0.05$)

marrow 6 days after exposure to bz or tce at three different doses and two exposure

average aneuploid frequency in hsc subpopulations from tce treated mice is 6%, regardless of exposure dose or schedule. Tce did not induce aneuploidy in lymphoid or myeloid subpopulations from exposed mice.

Hsc are located in the subpopulation that lacks lineage markers and expresses c-kit and sca (i.e., c-kit⁺sca⁺lin⁻), which comprises $0.29 \pm 0.05\%$ ($n=5$) of control marrow cells. The frequency of immunophenotypically-discriminated hsc in marrow from all bz or tce dose groups are $0.27 \pm 0.14\%$ ($n=4$) and $0.6\% \pm 0.29\%$ ($n=5$), respectively. The relative size of hsc containing populations, discriminated using immunophenotype, remained within the normal range after bz and tce.

The toxicity of topically applied bz and tce to hsc and progenitor populations has not been reported. Chemical toxicity to hematopoietic subsets was evaluated using CFU-GM assays in methylcellulose and limiting dilution cobblestone area forming cell (CAFC) assays. The average number of CFU-GM present in unseparated bone marrow from control animals is $7.3 \times 10^4 \pm 2.2 \times 10^4$ per femur+tibia. The CFU-GM frequency in unseparated

protocols approximates control values. Hsc from bz and tce treated mice were capable of generating similar numbers of CFU-GM as controls, indicating that these chemicals did not affect the ability of hsc to generate progeny committed to the myeloid lineages. While the CFU-GM assay measures progenitors committed to granulocyte/ macrophage differentiation, the CAFC limiting dilution assay probes a broad spectrum of immature hematopoietic subpopulations, including hsc. CAFC enumerated at increasing culture duration reflect increasingly primitive populations. The average CAFC value per femur+tibia in control marrow at d7, 14, 21 and 28 are 3.56×10^4 , 1.45×10^4 , 3.7×10^3 and 1.2×10^3 , respectively. CAFC enumerated at d7 and 21 were reduced 0.5 wk after mice received a single bz dose (65 and 40% of control, respectively, Figure 4a), while d14, 21 and 28 CAFC are reduced significantly 0.5 wk after multiple bz exposures (48, 16 and 26% of control, respectively, Figure 6a). CAFC frequencies at 1 and 2 weeks after bz exposure are only reduced at the d7 enumeration point for both single and multiple exposures (21 – 52% of control, Figure 6b, c). The results suggest that bz induces transient hematotoxicity in both primitive and mature subpopulations, as well as long-term hematotoxicity to the more differentiated cell types. In contrast, single or multiple exposures to tce (28 mg/kg) exposures did not affect the CAFC frequency at any measurement period (data not shown).

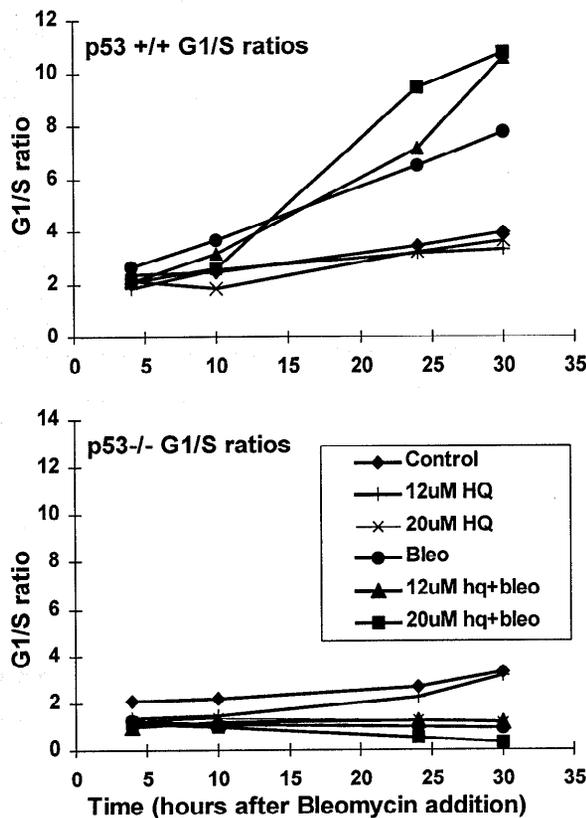
b) Bz exposure and cell cycle checkpoint measurements.

It has been suggested that induction of genomic instability is a critical step in development of the malignant phenotype. We postulated that bz and tce would induce genomic instability in hsc. We planned to test this hypothesis using previously described assays that demonstrated genomic instability in cultured fibroblasts and epithelial cells (16,33). These assays are based upon cellular responses to DNA damage that include activation of cell cycle checkpoints that prevent cell transition from G1-to-S, S-to-G2 and G2-to-M, until DNA damage is repaired. Failure to arrest allows genetically damaged DNA to be propagated to daughter cells, thus increasing accumulation of genomic aberrations. Inactivation of cell cycle checkpoints has been observed in multiple tumor cell lines, which fail to arrest at the G1/S or G2/M boundaries following exposure to DNA damaging agents (16). Cells with inactivated cell cycle checkpoints are able to overcome cell cycle arrest induced by agents such as N-(phosphonoacetyl)-L-aspartate (PALA) or bleomycin (bleo) and proliferate in the presence of these agents. We used these assays to evaluate the ability of bz and its metabolites to inactivate cell cycle checkpoints in marrow progenitors and marrow-derived stromal cells from mice with homozygous and heterozygous deletion of p53, a cell cycle checkpoint regulator, and mice with intact p53. The experimental design involved exposing hemopoietic and stromal cells to hydroquinone, a benzene metabolite, and subsequently challenging the cells with PALA or bleo. Several problems were encountered during these studies. It became apparent that the checkpoint assays that had been developed and applied using cell lines were not easily extended to primary cells. Initially, we planned to use standard myeloid progenitor assays as a readout system to detect PALA- resistant colonies. However, the semi-solid nature of the CFU-GM methylcellulose medium precluded repeated addition of PALA needed to maintain high PALA concentrations and progenitors proliferated even at relatively high initial doses (500 μ M). PALA (0.4

µg/ml) was cytotoxic to hemopoietic cells cultured in long-term assays, however, the culture systems were unable to maintain substantial hsc proliferation for sufficient periods to allow PALA resistance to emerge (which occurs over several weeks), even in cells from p53^{-/-} mice. Several modifications of the cell culture systems were attempted, but evaluation of checkpoints in hsc using PALA resistance will be dependent upon assays that allow long-term hsc proliferation, an area of investigation in numerous other laboratories.

Bone marrow derived stromal cells regulate hematopoiesis and hsc proliferation and can be maintained *in vitro* for weeks-to-months, thereby meeting several of the criteria required for the checkpoint assays. Primary stromal cell cultures were established and cells exposed to the test agent (hydroquinone) for approximately one day, followed by exposure to bleo, as described by Gonzales et al (33). The fraction of cells that incorporated bromodeoxyuridine (BrdUrd) from mice with and without intact p53 was quantified (Figure 7). An increase in the G1/S ratio after bleo would indicate G₁ arrest,

Figure 7. G1 checkpoint inactivation analyses



whereas a reduction would reflect checkpoint attenuation. Similar G1/S ratios in cells with intact p53 cells that were exposed to bleo with and without hydroquinone pre-exposure were observed, suggesting that bz metabolites do not attenuate checkpoints (Fig 7). Similar experiments in p53^{-/-} mice demonstrated that neither bleo nor hydroquinone affected G1/S ratios demonstrating that if checkpoints had been inactivated, they would have been detected in the readout system (Fig 7).

The cell cycle checkpoint studies suggest that checkpoint inactivation does not occur with bz metabolites. It is possible that additional bz induced genomic aberrations (i.e., additional genetic hits) are needed to attenuate checkpoints, and may require more sustained exposures (i.e., via inhalation) in mouse strains susceptible to developing bz-induced

leukemia. Additionally, the S-phase fraction in the presence of hydroquinone is similar to controls, suggesting that bz metabolites do not directly induce cell cycling, thus suggesting that studies proposed in aim #3 to investigate effects of multiple doses on hsc cycling might not be fruitful.

c) Mouse models of leukemia

Elucidation of the significance of early genomic events that increase susceptibility to leukemia will require that mice develop leukemia after exposure to radiation, bz and tce. We felt that the next step in evaluating the significance of early genomic events that increase susceptibility mandated that mice develop leukemia after exposure to radiation, bz and tce. Inbred strains with varying susceptibility to radiation-induced tumors and bz induced leukemia have been reported. For example, SJL and CBA/ca mice are predisposed to developing leukemia after single radiation exposures (11,45) and Cronkite et al (46) reported that CBA mice develop myeloid leukemia after bz exposure. We established that a single dose of radiation (3 Gy) plus dexamethasone induced myeloid leukemia in 25% of SJL mice, which was lower than the 30-40% induction rate reported in other studies. However, we were unable to induce myeloid leukemia in CBA/ca or CBA/J mice exposed to radiation or benzene (multiple doses administered by gavage). These latter results were disappointing in view of published reports of leukemia induction. The basis for lack of leukemia development is unknown, although anecdotal comments from other investigators also indicate difficulties in establishing bz induced leukemias in CBA mice. Nevertheless, leukemia development in irradiated SJL mice suggest that this strain will be useful for future studies to investigate the cooperative role of radiation and bz induced hsc genomic aberrations in leukemogenesis.

Relevance, Impact and Technology Transfer

DOE facilities and waste sites in the U.S are contaminated with mixtures of potentially hazardous chemicals such as metals, organic ligands, solvents, fuel hydrocarbons, polychlorinated biphenyls and radioactive isotopes. Epidemiological studies indicate that some of these chemicals, and radiation, are associated with increased risk of developing hematologic malignancies. However, uncertainties in exposure dose estimation and variation among individuals in innate susceptibility to tumor initiation and progression complicate biomonitoring and risk assessment. Limited knowledge about mechanisms underlying chemical induced carcinogenesis and the role of unrepaired DNA damage in this process further complicate evaluation of the leukemogenic potential of chemicals and radiation, particularly when present in mixed waste. Increased understanding of the genotoxic, cytotoxic and carcinogenic potential of chemicals and radiation is important to assess potential adverse health risks in occupational and environmental exposures. Data generated in this project have advanced understanding of chemical/radiation effects on the genome of hematopoietic stem cells, believed to be a target cell population involved in transformation to leukemia and have lend insight into biomarkers assays for exposure dose estimation.

Mechanisms of Leukemogenesis. Data generated in this project established that radiation, tce and bz exposure induce genomic aberrations in hsc, which are one of the cell types likely to be involved in malignant progression. We demonstrated that hsc with

aberrant genomes persist for long time periods in exposed animals and generate populations of genetically aberrant cells in the circulation and other hematopoietic tissues. Theoretically, these aberrant cell populations, which now carry genomic aberrations, are at increased risk of transformation if additional hits in critical regulatory pathways accumulate. These data suggest that even single chemical exposures (albeit at high doses), induce genomic damage, that may increase susceptibility to transformation. Confirmation that multiple exposures to radiation and chemicals increase risk of leukemogenesis will require an animal model that links induced genomic damage in hsc with leukemia susceptibility. It also appears that bz and its metabolites do not attenuate cell cycle checkpoints in primary hematopoietic cells, a common mechanism believed to contribute to genomic instability. Thus, other mechanisms (i.e., accumulation of additional unrepaired genomic damage, possibly resulting from long-term genotoxin exposure or unrepaired errors that occur during normal DNA replication, immune competence, etc.) are likely to be involved. Larger scale studies that evaluate low dose tce, bz and radiation genotoxic and immunotoxic effects in genetically engineered mice with targeted disruption/dysregulation in pathways thought to be involved in carcinogenesis are needed to evaluate the potential of these agents to synergize with inherited or acquired susceptibility to leukemogenesis.

Biomarkers

Our data raise important considerations for use of the FISH-based tfr assay in radiation dosimetry and biomonitoring. We demonstrate that the tfr is chromosome dependent at long times after irradiation. Preferential chromosome-specific radiation-induced TLs will impact radiation dose estimation unless these preferences are taken into account. Our data indicate that the assumption that TLs in circulating blood cells reflect frequencies of genetically aberrant hsc is likely to be true. Thus, quantification of the frequency of circulating reporter populations with translocations provides reasonable biomarkers for *in vivo* radiation dose estimation at early times after exposure. However, at later time periods the frequency of aberrant cells in the circulation fluctuates dramatically between individuals that received the same dose, suggesting variation among individuals in the clonal expansion of genetically aberrant cells. These variations complicate dose estimation using TL biomarkers, but may lend insight into some of the early events leading to clonal expansion, often observed in pre-leukemia conditions, such as myelodysplasia.

The relationship between chemical exposure and genomic damage in hsc and differentiated cells in the circulation was also defined. The frequency of hsc with aneusomies after single and multiple exposures to bz and tce is increased significantly. However, aneusomy in mature lymphocytes and myeloid subpopulations from bz and tce treated mice are variable. In contrast to radiation exposures, there is no apparent dose response relationship between bz and tce dose and frequency of aneuploid hsc or circulating myeloid or lymphoid populations *in vivo*. The lack of dose response relationship *in vivo* indicates that aneuploid cells in the circulation or in the bone marrow are not useful biomarkers for to estimate chemical exposure dose.

Project Productivity

The overall goal of this project to establish linkage between chemical/radiation exposure and induction of genomic damage in target populations with the capability to undergo transformation was accomplished. Two of the three aims were completed. The third aim is in progress as we seek funding to complete studies to examine radiation/bz/tce interactions that increase genomic damage in hsc and establish a link between damage incurred and leukemogenesis. Completion of the third aim was limited by failure to demonstrate bz induced leukemias in mice. The basis for lack of leukemia induction is unclear, although other investigators have experienced similar difficulties.

Personnel Supported

M.G. Pallavicini, Ph.D.	PI	partial support
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L. Grewal, M.S.	Technician	Partial support
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Publications

Peer-reviewed

Giver CR, Wong R, Moore II DH and Pallavicini MG. Radiation- Induced Translocation Biomarkers in Inbred Mice with Varying Cancer Predisposition (2000) *Radiation Res (In Press)*

Giver CR, Wong R, Moore II DH and Pallavicini MG. Benzene and Trichlorethylene Induce Genomic Aberrations in Hematopoietic Stem Cell Subpopulations. Submitted, 2000.

Giver CR and Pallavicini, MG. Long Term Persistence of Bz Induced Aneusomies in Hematopoietic Stem Cells In preparation, 2000.

Pallavicini MG, Grewal L, Dharia A, and Wong R. Lifetime Persistence of Radiation Induced Translocations in Hemopoietic Stem Cells. In preparation, 2000.

Interactions

1997 Lawrence Berkeley Laboratory: Hematopoietic Stem Cells as Targets for Radiation Induced Genomic Damage

1997 Emory University Grand Rounds: Leukemia and Hematopoietic Stem Cells

1998 UCSF Cancer Center: Hematopoietic Stem Cells and Leukemogenesis

1999 Rush Presbyterian Hospital Environmental Contaminant Induced Genomic Damage in Hematopoietic Stem Cells

Presentations at Meetings/Congresses

1998 EMSP, Chicago Poster Presentations: Radiation and chemical induced genomic damage in hematopoietic cells *in vivo*.

Radiation-induced translocation biomarkers: assumptions tested using inbred mice with varying cancer predisposition. Cynthia R. Giver, Dan Moore II, Maria G. Pallavicini. American Society of Hematology meeting, December 1999, New Orleans, LA.

Benzene-induced toxicity and aneusomy in murine bone marrow hemopoietic stem cells. Rosemary Wong, Cynthia R. Giver, and Maria Pallavicini. NIEHS meeting "The Role of Human Exposure Assessment in the Prevention of Environmental Disease. September 1999, Rockville, MD.

Benzene-induced toxicity and aneusomy in murine bone marrow hemopoietic stem cells. Rosemary Wong, Cynthia R. Giver, and Maria Pallavicini. American Society of Hematology meeting. December 3-7, 1999, New Orleans, LA.

Transitions

NIH. Data generated in this project provides baseline information for a funded project to evaluate radiation, bz and tce effects on human hematopoietic stem cells transplanted into immune-deficient mice

Patents

None

Future Work

Data from this project have demonstrated that radiation and chemical exposure can alter the genome of hsc and that genetically aberrant hsc persist throughout lifetime hematopoiesis. While these genomic alterations did not result in leukemia, it is possible that more prolonged exposures (weeks to months) may induce additional aberrations that

increase susceptibility to malignancy. This possibility should be evaluated, but will require relatively large numbers of mice. Since we have shown that bz does not inactivate checkpoints, other mechanisms (interruption of pathways that regulate cell survival, DNA repair, apoptosis, immune surveillance, etc.) should be explored to determine whether chemical/radiation induced genomic aberrations synergize with known genetic defects to increase leukemia susceptibility. This is best accomplished using genetically engineered mice with perturbations in candidate regulatory pathways.

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Feedback

None

Appendices

Two manuscripts