UNIVERSITY OF OTTAWA

Faculty of Medicine **Faculty of Health Sciences**

Benzene State of the Science Workshop
December 16 & 17, 1998

Institute of Population Health
Faculty of Medicine and Faculty of Health Sciences

Agenda – December 16, 1998
Benzene Workshop
University of Ottawa • Institute of Population Health

8:30 am	Welcome	Daniel Krewski Rapporteur
8:45 am	Overview of the Benzene Problem	Robert Snyder
	Benzene Metabolism and Genotoxicity, Bob Sonawane, Chair	
9:15 am	Benzene Metabolism	David Ross
9:35 am		Paul M. Schlosser, Discussant
9:45 am		Open Discussion
10:00 am	Break	
10:15 am	Benzene-induced Genotoxicity	John Whysner
10:35 am		David A. Eastmond, Discussant
10:45 am		Open Discussion
11:00 am	Implications for Risk Assessment	Daniel Krewski, Rapporteur
	Toxicity & Leukemogenesis, Patrick Beatty, Chair	
11:15 am	Value of Transgenic models	John E. French
11:35 am		Robert Snyder, Discussant
11:40 am		Discussion
12:00 pm	Lunch	
1:10 pm	Hematotoxicity Modeling	Patrick Beatty
		Melvin Andersen
1:30 pm	Secondary Leukemogenesis	Richard A. Larson
1:50 pm		Armand Keating, Discussant
2:00 pm		Discussion
2:15 pm	Hematotoxicity & Leukemogenesis	Azra Raza
2:35 pm		Debra L. Laskin, Discussant
2:45 pm		Discussion
3:00 pm	Break	
3:15 pm	Mechanism of Leukemogenesis	Richard D. Irons
3:35 pm		George F. Kalf
3:55 pm		Martyn T. Smith, Discussant
4:05 pm		Discussion
4:25 pm	Implications for Risk Assessment	Daniel Krewski, Rapporteur
4:40 pm		Discussion
5:00 pm	Conclude	
7:00 pm	Dinner; Dinner speaker: Ron Worton, University of Ottawa	

Agenda – December 17, 1998
Benzene Workshop
University of Ottawa ● Institute of Population Health

Benzene Induced Leukemia: Human Studies, Carol Henry, Chair

8:30 am	NCI/Chinese Epidemiology Study	Richard B. Hayes/Martha Linet
8:50 am	Insights from Petroleum Studies	Robert Schnatter
9:10 am	Biomarkers of Leukemia Risk	Martyn T. Smith
9:30 am	HEI Biomarker Research in China	Martha E. Richmond
9:50 am	New Initiatives for Epidemiologic Research	Otto Wong
10:10 am	Break	
10:30 am	Discussion Panel	Jack Siemiatycki Pierre Band David Bayliss Bob Sonawane
11:15 am	Open Discussion	
11:55 am	Implications for Risk Assessment	Daniel Krewski, Rapporteur
12:10 pm	Lunch	

Future Research to Improve Risk Assessment & Risk Management, Bernie Goldstein, Chair

1:00 pm	U.S. EPA Perspective	Bruce Rodan
1:20 pm	Health Canada Perspective	Bette Meek
1:40 pm	European Union Perspective	E. Dinant Kroese
2:00 pm	California EPA Perspective	Lauren Zeise
2:20 pm	Petroleum Industry Perspective	Patrick Beatty Geoffrey Granville
2:40 pm	Good Science for Good Decisions; The NIEHS Perspective	Christopher Schoenwalder
3:00 pm	Open Discussion	
3:45 pm	Concluding Remarks	Daniel Krewski, Rapporteur
4:00 pm	Conclude	

1998 Benzene State of the Science Workshop
University of Ottawa, Institute for Population Health

Observations for Presenters

Thank you for agreeing to participate in this important workshop. Our goal for the two days we will be together is to establish a road-map for future benzene research. As you are already aware, the purposes of this workshop include:

- reviewing the current state of knowledge about benzene leukemogenesis
- identifying data gaps and areas where further research would contribute meaningfully to scientific understanding of the mechanism and extent of benzene's human hematotoxicity and leukemogenesis
- understanding the implications of the benzene database for risk assessment and for the regulatory context in which the science is used.

The following questions are intended to assist you in focusing your thinking about what should be presented at the workshop in Ottawa, and about the direction your discussion should pursue. Our purpose is to focus your thoughts on the present state of the science and the future direction research should be directed. We also want to help you maintain the broader perspective of the application of this research to the risk assessment process.

Benzene Metabolism and Genotoxicity - B. Sonawane, Chair

Benzene Metabolism

How can estimates of the leukemogenic risk of benzene be improved by knowledge of:

1.) The identity of the leukemogenic metabolite(s) of benzene.
2.) Target tissue levels of benzene metabolites.
3.) The enzymology of benzene metabolism.
4.) The role of metabolites transported to the bone marrow from other organs vs. in situ formation.

In the above, presenters might consider the implications for dose-response, biomarkers of exposure, possible mechanisms of hematotoxicity and genotoxicity, and inter-individual variation in hematotoxic and leukemogenic response. In what experimental systems can useful information be developed, e.g. experimental animal studies, human *in vitro*, studies of exposed human populations?

Benzene-Induced Genotoxicity

Although the weight of the experimental evidence strongly suggests that benzene does not induce point mutations, there are data from multiple techniques which, suggest that benzene derived material binds to genetic material. Numerous studies have linked benzene exposure with chromosomal aberrations and certain chromosomal rearrangements are associated with AML. How important to benzene risk assessment is distinguishing between direct DNA binding of benzene and chromosomal rearrangements

as critical to the leukemogenic process? What are the implications for the dose response characteristics of benzene-induced leukemia, and how might this question be answered?

To what extent can specific chromosomal rearrangements be identified as specific to leukemia secondary—to chemical exposures as opposed to *de novo* leukemia?

What are the implications for risk assessment of various proposed mechanisms of benzene-induced clastogenicity, e.g. DNA- protein crosslinks, interference with mitotic apparatus, topoisomerase inhibition.

Toxicity & Leukemogenesis - P. Beatty, Chair

Some of the core questions that may be addressed in this session include:

1.) What are the mechanisms by which reactive metabolites of benzene influence bone marrow function leading to aplastic anemia?
2.) What are the mechanisms by which reactive metabolites of benzene influence genotoxicity, chromosome damage, and transformation to a neoplastic cell?
3.) Does benzene-induced leukemia develop through a series of steps similar to the classic carcinogenic model, i.e. initiation, promotion, progression?

Hematotoxicity and Leukemogenesis

At least one multi-factorial model of benzene induced leukemia has been proposed which includes an interplay of both genotoxic and non-genotoxic mechanisms. Based upon a model of this basic form:

1.) By what mechanisms could hematotoxicity play a role in the induction of leukemia, e.g., effects on apoptosis or alteration of proliferation or differentiation of critical cell types?
2.) Is hematotoxicity (as determined by what endpoint) an obligatory part of the leukemogenic process?

What are the implications for risk assessment of the involvement of hematotoxicity in the leukemogenic process, e.g., shape of the dose-response, importance of peak versus cumulative exposures for risk?

Hematotoxicity Modeling

This presentation is expected to summarize the modeling workshop held the day before the State of the Science Workshop. In the summarization, a focus should be on how well the toxicodynamic model proposed by Cox describes the development of benzene toxicity or leukemogenesis, and how it could be used in the risk assessment process.

Secondary Leukemogenesis

A number of other chemicals are known to cause leukemia, primarily AML, secondary to exposure. Some of these compounds require metabolic activation while others are direct acting; some compounds are linked to specific biochemical mechanisms such as inhibition of topoisomerases while the leukemogenic mechanism of others is not readily apparent.

Can the actions of benzene on the bone marrow, such as the development of myelodysplasia, lead to the characterization of benzene as a "secondary leukemogen?" If so, where does benzene fit into this spectrum of secondary leukemogens, and can this information provide useful guidance for risk assessment

of benzene induced leukemia, e.g., leukemia cell type for quantitative potency calculations, relative roles of genotoxic vs. non-genotoxic mechanisms & dose-response implications?

Value of Transgenic Models

The history of research into benzene leukemogenesis has been plagued by the lack of an animal model for this endpoint. Initial reports indicate that oral dosing of benzene to a transgenic mouse strain, Tg.AC, containing an activated H-ras oncogene, results in induction of granulocytic leukemia. What additional information must be developed in order to validate this mouse strain as a model for benzene-induced leukemia in humans? What questions, raised in earlier sections of this document, may be experimentally accessible with this model?

Mechanism of Leukemogenesis

The biological mechanism of benzene-induced leukemogenesis has been suggested to be similar the model for colon cancer, a multi-component process with the involvement of both genetic and non-genetic mechanisms. Does the state of the science currently support such a model in concept? If not, what additional type(s) of information would confirm or disprove the model?

Elucidating the exact structure of a multi-component model in a complex biological system such as the hematopoietic system is, at best, a complex problem, and complete knowledge may be beyond the capabilities of current technology. Considering the implications for benzene's risk assessment of such a multi-component model, in what detail does the model have to be understood to provide useful guidance for risk assessment? For instance:

1.) Is it critical to issues such as dose response to know the temporal sequence of the genetic and non-genetic components?
2.) To what extent is knowledge of the exact identity of the components of the mechanism necessary in order identify relevant leukemia cell types for epidemiology studies?
3.) Is it enough to determine that oncogene activation is the genetic event in leukemia induction or is the identity of the oncogene(s) involved necessary?

Benzene Induced Leukemia: Human Studies – C. Henry, Chair

Epidemiologic Research

Exposure assessment is a common area of weakness in most occupational and environmental epidemiology studies. Problems often encountered include the need to estimate or model exposures from limited monitoring data, and the presence of other, potentially confounding, exposures that cannot be adequately controlled for. Recognizing exposure assessment as a fundamental issue for epidemiologic research into the leukemogenic effect of benzene, presenters may wish to consider:

1.) what are the limitations of exposure data from the studies being considered, and of the studies that have been used to date in regulatory risk assessment?
2.) Are there additional data on exposure which could be accessed and analyzed to enable more accurate quantification of exposure-response for existing studies, or for future planned studies?
3.) How might such information be accessed and analyzed?

4.) What is the minimum dataset for quantification of exposure in epidemiologic studies needed in order to accurately estimate dose-response, and hence to use the study in a risk assessment?
5.) Are there data available which would permit meaningful additional analysis to shed light on risks associated with peak versus cumulative exposure?

In considering potential confounding exposures and other co-factors, presenters may consider addressing issues such as:

1.) Are there data which would permit systematic investigation of substances other than benzene to which subjects in their studies may have been exposed, or may potentially be exposed, and how could these data be brought into play?
2.) How should exposures to other chemicals be considered in understanding the risk associated with exposure to benzene?
3.) Would it be possible or worthwhile to systematically investigate socioeconomic and ethnic differences which might contribute to variations in risk between North American and Chinese populations?
4.) Would additional analysis of confounders, such as smoking or familial history of cancer, contribute meaningfully to our knowledge base about risks in petroleum workers associated with exposure to benzene?

Other issues presenters may wish to consider in formulating their remarks include:

1.) What are the plausible scientific reasons for the variations in findings from the different studies? How plausible is it that these variations are due only to the variations of sensitivity of the different investigations?
2.) Is it feasible to investigate incidence/mortality due to acute myelogenous leukemia alone in further analyses of benzene-exposed cohorts? What is the importance of considering specific cell-types and is it feasible to do this using the epidemiologic method?

Biomarkers

Biomarkers present a potential opportunity to enhance the understanding of human health risks from benzene exposure. Presenters may wish to consider

1.) What is the technical feasibility of using biomarkers for benzene research?
2.) Are there ethical considerations in the use of biomarkers?
3.) How would benzene biomarkers relate to other biomarkers that have been identified?
4.) Are there correlations between biomarkers of exposure and biomarkers of effects, as they relate to benzene and leukemia?
5.) How could biomarkers be incorporated into epidemiologic research, and what potential do they have for strengthening the understanding of associations between benzene and health effects?
6.) Is there a way of attributing biomarkers to specific sources of exposures?
7.) How should biomarkers be utilized in the risk assessment process, and what weight should be ascribed to studies that use them?

Abstracts

Benzene State of the Science Workshop
December 16-&17, 1998

Overview of the toxicology of benzene. Robert Snyder, Ph.D., Professor and Chairman, Department of Pharmacology and Toxicology, Rutgers, the State University of New Jersey and Associate Director, Environmental and Occupational Health Sciences Institute, Piscataway, NJ, USA.

Santesson (1897) and Selling (1910), reported that chronic exposure to benzene in the workplace resulted in marked decreases in circulating blood cells and subsequent fatality. An overview of benzene-induced bone marrow depression can be derived from industrial studies where large groups of workers were exposed and various stages in the severity of the disease could be identified among the worker population, e.g., Helmer (1944), Greenburg et al. (1939), Hamilton-Patterson and Browning (1944), Savilahti (1956), Aksoy et al. (1971). Chronic exposure to low concentrations of benzene may produce reversible decreases in blood cell numbers. However, higher chronic exposures lead to the onset of irreversible bone marrow depression which is characterized by anemia, leucocytopenia, with emphasis on lymphocytopenia, and/or thrombocytopenia. Decreases in all three cell types is defined as pancytopenia, which in the case of benzene toxicity results from severely damaged bone marrow, and the disease is termed aplastic anemia. The recognition that benzene was a leukemogen was established on the basis of studies among exposed worker populations by in Italy by Vigliani and Forni (1976), in Turkey by Aksoy and Erdem (1978), in the United States by Infante et al. (1977) and in China by Yin et al. (1987).

Decreases in circulating blood cells may also be observed in the presence of a dysplastic marrow. Myelodysplastic syndrome (MDS) is a preleukemic state characterized by abnormal marrow architecture, inadequate hematopoiesis, and many cells demonstrating chromosome damage. MDS is a clonal disease (Janssen et al., 1989) which may result in response to chemotherapy by alkylating agents (Kantarjian and Keating., 1987) or to chronic benzene exposure (Forni and Moreo, 1967,1969; van den Berghe et al., 1979). Chronic benzene toxicity leading to MDS usually proceeds to acute myelocytic leukemia (AML). Chromosome damage has been associated with benzene-induced leukemia since the observations of Pollini et al. (1964) and Forni et al. (1971). Damage to chromosomes 5 and 7 have been associated with MDS (Jacobs et al., 1986) and AML (Golomb et al., 1982) and after benzene exposure (Sasiadek, 1992). Smith et al., (1998) have demonstrated translocations and aneusomy in chromosomes 8 and 21 after benzene exposure.

Whether the result of benzene exposure is aplastic anemia or leukemia, death usually results from infections because among the effects of benzene is depression of the immune system. Immunotoxic effects of benzene have been known since the early 1900's (Snyder and Kocsis, 1975). Benzene depresses B- and T-lymphocytes as well as their mitogenic responses (Rozen and Snyder, 1985). Lymphocytes are decreased both in the circulation and in the bone marrow in rats and mice (Wierda and Irons, 1982). Mice treated with benzene by subcutaneous injection displayed fewer peritoneal macrophages, decreased phagocytic and tumor cell cytolysis capability, and increased hydrogen peroxide generation (Klan et al., 1990). Within the bone marrow an increased number of macrophages, enhanced chemotaxis, elevated hydrogen peroxide production, as well as increases in mononuclear phagocytes were observed (MacEachern et al., 1992). Furthermore, nitric oxide production in bone marrow macrophages, which appears to be tied to benzene-induced decreases in hematopoiesis, is increased (Laskin et al., 1995). The data suggest that impairment of macrophage activity is mediated by benzene

metabolites (Lewis et al., 1988; Manning et al., 1994). The expression of impaired immune function was demonstrated by Rosenthal and Snyder (1985) who reported that benzene-exposed mice demonstrated decreased resistance to infection by L. monocytogenes.

The processes of differentiation and amplification which mark the progression from pluripotential stem cells through the various stages of maturation has an absolute requirement for a functional hematopoietic microenvironment. This system, referred to as the stromal cells of the bone marrow, includes macrophages, endothelial cells, reticular cells, fat cells, etc. These cells provide nutrients, growth factors and an environment necessary for normal bone marrow function. Using a technique which involves long term bone marrow cell cultures, Garnett et al. (1983) showed that stroma from benzene treated mice were ineffective in supporting the differentiation of stem cells from untreated mice. Gaido and Weirda (1984,1985,1987) reported that hydroquinone and benzoquinone decreased the ability of stromal cells to support G/M colony formation. Stromal macrophage production of IL-1 was inhibited by hydroquinone (Renz and Kalf (1991). A hypothesis worth considering is that damage to the stroma may be the most significant factor in the decrease in circulating cells that is ultimately termed aplastic anemia.

Thus, benzene toxicity is a continuum of events leading from decreases in circulating blood cells, to pancytopenia and aplastic anemia or to MDS and acute non-lymphocytic leukemia. Chronic exposure is necessary, but the limits on the "dose X time" relationship are only partially understood. Because of the impairment of the immune system, death may result as a function of marrow dysplasia or aplasia, or in leukemia.

Benzene was one of the prototype compounds which led to the understanding that toxicity of organs other than liver may be the result of metabolites formed in the liver , as well as, in target organs. Inhibition of benzene metabolism (Andrews et al., 1977) or partial hepatectomy (Sammet et al., 1979) protected against benzene-induced decreases in erythropoiesis. All of the known unconjugated metabolites of benzene, with exception of phenol and 1,2,4-benzenetriol have been shown to decrease erythropoiesis (Snyder and Hedli, 1996). Furthermore, the evidence indicates that the toxic effects result from interaction between benzene metabolites. Eastmond et al. (1987) reported that phenol and hydroquinone interacted to exacerbate decreases in bone marrow cellularity and Guy et al. (1990) showed that combinations of either phenol plus hydroquinone, or phenol plus catechol, were more hematotoxic than any of the metabolites given alone. The most potent and effective combination in inhibiting erythropoiesis is hydroquinone plus muconaldehyde (Snyder et al., 1989).

There appear to be several mechanisms by which benzene metabolites impair cellular functions. For example, Irons and Neptun (1980) suggested that hydroquinone derived from benzene covalently binds to tubulin, a protein essential for spindle formation in mitosis, and thereby, inhibits cell replication. Schwartz et al. (1985) demonstrated that benzene metabolites inhibit mitochondrial DNA polymerase. Kalf et al., (1996) reported that p-benzoquinone inhibits the conversion of pre-interleukeins-1 and -1 to the active cytokine. Snyder et al. (1978) found that benzene metabolites were covalently bound to protein in several body organs after treating mice with radiolabeled benzene and Lindstrom et al. (1998) reported on specific adducts of benzene oxide with hemoglobin and albumin in mice, rats and humans. Rushmore

et al.(1984) demonstrated that benzene metabolites covalently bind to mitochondrial DNA and inhibit mitochondrial RNA synthesis.

Damage to nuclear DNA by benzene metabolites has been studied in several laboratories. Lutz and Schlatter (1977) observed benzene metabolites bound to hepatic DNA after exposing animals to radiolabeled benzene by inhalation. Structures of benzoquinone adducts to guanine, adenine and cytosine have been proposed (Jowa et al., 1990; Pongracz et al., 1991). DNA adducts arising from benzene have been measured in HL-60 cells (Hedli et al., 1996; Levay et al., 1992; 1993). Although there have been reports of DNA adducts from benzene in vivo, the level of adduct formation is not consistent and when reported is at a very low level. Thus, Reddy et al. (1994) failed to detect DNA adducts in benzene treated mice using the [^{32}P]post labeling assay, whereas Creek et al. (1997), using accelerator mass spectrometry reported observing adducts to both protein and DNA in mice given [^{14}C]benzene in the ng/kg range.

An alternative hypothesis for DNA damage is oxidation of DNA by reactive oxygen species that arise as a result of benzene metabolites. For example, p-benzoquinone and hydroquinone increase superoxide, nitric oxide, and hydrogen peroxide in HL-60 cells activated with phorbol ester (Rao and Snyder, 1995). Boersma et al. (1994) suggested that hydroquinone itself is unlikely to generate reactive oxygen species. However, Brunmark and Cadenas (1988) reported on a metabolic pathway from hydroquinone leading to the production of glutathionyl-benzenetriol, which can undergo autooxidation leading to superoxide formation.

An interesting corollary mechanism, proposed by G. Rao (1994) suggests that benzene induced DNA damage is mediated by (1) release of free iron in the bone marrow of benzene-treated animals [probably by polyphenolic metabolites of benzene], followed by (2) the chelation of iron by hydroquinone or benzenetriol to yield (3) a reactive oxygen-generating species such as superoxide, which in turn (4) causes oxidative damage to DNA. His suggestion that glutathionyl hydroquinone may be a key intermediate (Rao, 1996) resonates well with the hypothesis of Brunmark and Cadenas 1988). In a radical rich system glutathionyl-hydroquinone may well be converted to glutathionyl-benzenetriol. In Singh et al. (1994) he suggests a chemical structure for the iron-benzenetriol chelate in which the iron is bound between the hydroxyl groups at positions 1 and 2 on 1,2,4-benzenetriol with the release of two protons. Indeed, it may well be that the autooxidation of glutathionyl-benzenetriol is enhanced with an iron chelated between positions 1 and 2. A synthesis of the ideas of Rao et al. and Brunmark and Cadenas suggests that the formation and autooxidation of glutathionyl-benzenetriol yields superoxide. The iron- benzenetriol chelate mediates the conversion of superoxide to singlet oxygen and hydroxyl radical which can hydroxylate guanine in the 8 position. Richter (1997) suggested that, although 8-hydroxyguanine is the most frequently observed oxidation product of DNA, any of the DNA bases may undergo hydroxylation, and potentially result in a mutational event.

An alternative action which may sensitize bone marrow cells is the ability of some benzene metabolites to inhibit topoisomerase II. Chen and Eastmond (1995) and Hutt

and Kalf (1996) showed that p-benzoquinone was a topoisomerase II inhibitor. They suggest that compounds having similar inhibitory activity when used to treat cancers can cause subsequent delayed leukemia.

In studies of the response of bone marrow cells to hydroquinone, Irons and Stillman (1996) using human CD34+ cells reported enhanced clonogenic responses to GM-CSF. Hazel et al., (1996) using mouse 32D.3 myeloblast cells reported initiation of differentiation by hydroquinone and benzene, which terminated at the myelocyte stage and implicated a role for hydroquinone at the leukotriene D4 receptor (Hazel et al., 1995). Hydroquinone can also inhibit apoptosis at the myeloblast/myelocyte stage of differentiation (Hazel et al.,1996). These effects provide a mechanism for large numbers of immature white blood cells to accumulate. In the event that one of the cells entering the expanding myelocyte pool has undergone a leukemogenic transformation at an earlier stage in maturation, it may serve as the parent of an expanding clone of leukemia cells.

Given that benzene metabolites may cause DNA damage leading to a mutagenic effect, which may be reflected as a translocation, or other DNA damage such as that related to inhibited topoisomerase II, the process may be termed "initiation". The effects of benzene and hydroquinone leading to expansion of a less than fully differentiated pool of myeloid cells, provide a mechanism of "promotion". Thus, one can visualize a two step process of initiation and promotion associated with benzene-induced non-lymphocytic leukemia.

The major problem that remains in our attempts to understand the mechanism of benzene toxicity/leukemogenesis is the interplay between exposure and sensitivity. The measurement of exposure to benzene, which defines the dose, has been difficult to achieve in retrospective studies, and dose reconstructions, usually of questionable accuracy, is required. Sensitivity refers to factors which may render an individual sensitive or resistant to benzene. An incomplete list of variable factors includes hepatic CYP 2E1 (Rothman et al., 1997) activity because it is the major enzyme involved in benzene hydroxylation, the activity of conjugating enzymes of which the glutathione transferases seem to be significant, and comparative activity of myeloperoxidase (London et al., 1997) and reductase (NQ01) (Rothman et al., 1997) in bone marrow. Attempts to extrapolate effects at high dose to those at low dose must take these factors into account. The issues that underlie chronic, as opposed to acute toxicity, must also be carefully explored. Whereas, in the past the product of dose times time was thought to be a constant, it is now recognized that either factor may best be described by exponential functions. It is only through a thorough understanding of the mechanism of action that we can appreciate the dose response relationship.

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Metabolic Mechanisms of Benzene Toxicity. David Ross, Molecular Toxicology and Environmental Health Sciences Program, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO, USA.

A number of recent reviews have been published on benzene metabolism which provide an overview of the process (1-3). The importance of benzene metabolism to toxicity is supported by inhibition of benzene toxicity by toluene, a competitive inhibitor of benzene metabolism (4), the reduced toxicity of benzene in animals which have undergone partial hepatectomy (5) and the reduction of benzene toxicity in CYP2E1 knockout mice (6).

The potential relevance of putative toxic metabolites for the target organ toxicity of benzene and their sites of generation will be discussed. Metabolic mechanisms postulated as responsible for benzene toxicity include the generation of i) benzene oxide ii) open ringed metabolites such as trans trans muconaldehyde (MA) , iii) polyphenolic metabolites such as hydroquinone, catechol, 1,2,4-trihydroxybenzene and their quinone oxidation products and iv) combinations of metabolites.

Benzene oxide is formed during metabolism of benzene in mouse, rat and human liver microsomes (7, 8) and can be measured in the blood of rats after administration of benzene (9). Protein adducts of benzene oxide have been detected in the blood and bone marrow of mice and rats exposed to benzene (10). In a recent study (11) hemoglobin and albumin adducts of benzene oxide were detected in workers exposed to high levels of benzene. MA is formed during metabolism of benzene in mouse hepatic microsomes (12), via mechanisms which have been suggested to involve singlet oxygen or hydroxyl radical mediated oxidation of benzene or ring opening of the hydroxylated oxepin derived from benzene oxide (13, 14). MA has not been isolated in-vivo after benzene administration although trans trans muconic acid is a metabolite of benzene in animals and humans (15,16). MA and its OH/CHO derivative are both hematotoxic (13) and cytotoxic (17). Distribution studies have demonstrated that a small percentage of 14C-MA administered to mice (ip or iv) reached the bone marrow (18).

Phenolic metabolites of benzene are major hepatic metabolites of benzene which may undergo Phase II conjugation reactions (19). Alternatively, benzene-derived phenolics may be released into the blood (20, 21) and migrate to bone marrow (22). Distribution of conjugated phenolics to distal sites followed by deconjugation also represents a possible mechanism of delivery of phenolic metabolites to extrahepatic target organs (23). We have been unable to detect cytochrome P4502E1 in human bone marrow by immunoblotting but marrow is rich in myeloperoxidase which can convert polyphenolics into reactive quinones. Interestingly, other target organs of benzene

toxicity also contain significant peroxidase activity (23,24). Quinones can either arylate biological nucleophiles or redox cycle generating reactive oxygen species and oxidative stress. Derivatives of quinones may also be responsible for toxicity. Glutathione adducts of 1,4-benzoquinone, for example, are known to be redox active and have been demonstrated in the bone marrow of mice after administration of benzene and are hematotoxic when administered to mice (25). Polyphenolic metabolites of benzene are not necessarily innocuous species and may exert mutagenic (26) and deleterious effects, such as inhibition of ribonucleotide reductase (27), independent of their oxidation to quinones. Combinations of benzene metabolites have also been suggested to play a role in benzene toxicity (1, 28-30). Since many reactive metabolites may be formed during benzene metabolism, metabolic mechanisms underlying benzene toxicity may well be multifactorial and this evidence will be summarized.

Recent work has provided evidence for metabolic susceptibility factors for benzene toxicity in humans (31). Occupationally exposed individuals with a higher capacity for cytochrome P4502E1 mediated metabolism and a lack of NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase), due to a polymorphism in the NQO1 gene, have an increased susceptibility to the hematotoxic effects of benzene. NQO1 may function to keep benzene-derived quinones in a reduced form where they can be detoxified by phase II conjugation reactions or by maintaining antioxidant levels of ubiquinone or a-tocopherol (32)

Within the bone marrow both hematopoietic progenitor cells and stromal cells are potential targets of benzene toxicity. The CD34+ progenitor compartment contains all short term and long term repopulating cells in marrow and progenitor cells are considered likely initial target cells in leukemias. CD34+ cells contain appreciable myeloperoxidase (33, 34) and phenolic metabolites of benzene have been reported to induce apoptosis in human marrow CD34+ cells (35). The stroma provides a supporting framework within the medullary cavity for blood cell development and has been implicated as a target of the toxicity of benzene metabolites. Levels of myeloperoxidase, NQO1 and glutathione have been suggested as metabolic determinants of the effects of benzene-derived phenolics in bone marrow stroma (36, 37).

The characterization of a single benzene metabolite or a combination of metabolites which is/are responsible for the pathological effects of benzene has not been possible to date. This is important for the purposes of risk assessment and for studies designed to elucidate the pathogenesis of benzene-induced bone marrow damage. Evidence supporting the role of either benzene epoxide, open ringed metabolites, phenols/quinones or combinations of these metabolites as putative toxic metabolites of benzene will be discussed as will problems associated with each potential metabolic mechanism. It is

essential to assess the biological plausibility of selective toxicity at the level of the bone marrow for each potential mechanism. Significant data gaps in studies of benzene metabolism and areas for future work will be identified. Particularly, the use of stably transfected cell lines and transgenic animals represent valuable techniques that can answer questions regarding the metabolic mechanisms underlying benzene toxicity.

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Needs for Research on Benzene Metabolism and Dosimetry

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In the past, benzene was used extensively in the production of paints, resins, rubber, inks, and dyes. Evidence that persons exposed to high levels of benzene for extended periods of time suffer an increased risk of aplastic anemia and acute myelogenous leukemia (AML) (1) prompted the adoption of regulations by the U.S. government that limited occupational exposures to benzene (2). Recent reports of increased hematotoxicity and leukemia among benzene-exposed workers in China corroborate the existing evidence that benzene causes both aplastic anemia and AML (3,4). As in previous studies, AML in the Chinese cohort was associated with constant, high-level exposures or high cumulative exposures.

Benzene is found in gasoline at a relative abundance of 1% by volume and is used in the as a feedstock for the synthesis of many organic chemicals (5). Common sources of environmental exposure include gasoline fumes, automobile exhaust, and both mainstream and side stream tobacco smoke (6). A clear concern is whether or not these ubiquitous, low-level exposure present a significant health-risk to humans for AML, and what level of exposure can be considered to be of negligible risk. From a risk-management viewpoint, the purpose in understanding and quantifying the dosimetry and metabolism of benzene and its metabolites would be to use this information in characterizing the exposure-dose relationship for benzene. In particular, one would like to know how the concentration of key metabolites in bone marrow, including phenol, hydroquinone, muconaldehyde, and possibly benzene oxide, relates to the level of benzene to which an individual is exposed. These metabolites (with the possible exception of benzene oxide, the effects of which have not been investigated)

appear to play an important role in benzene-induced myelotoxicity (7, 8) and likely are significant in the induction of AML. Therefore, any nonlinearity in the exposure-dose relationship for these metabolites would be expected, in turn, to lead to nonlinearity in the exposure-response relationship for benzene-induced AML. Hence, determining or quantifying the shape of these exposure-dose relationships should improve estimates of the exposure-response relationship, particularly in extrapolating from high-level exposures at which benzene-induced AML is observed to low-level exposures of current concern.

There has been considerable effort to characterize the metabolism and disposition of benzene and its metabolites in rodents (e.g., 9-13) as well as some work in non-human primates (14). These laboratory animal data are useful currently in that they can allow one to develop and validate physiologically-based pharmacokinetic (PBPK) models in animals before attempting to extrapolate such models to humans. Further, one could potentially use rodent PBPK models to examine the relationship between benzene dosimetry and benzene-induced effects in rodents that might be considered precursor events and/or representative of human risks for AML. However, since there is not currently a confirmed animal model for AML induced by inhalation exposure to benzene, such modeling efforts are of limited utility beyond validating the PBPK model-development process.

There are two notable exceptions to the completeness of the data set for rodents. The first is the dosimetry of *trans-trans*-muconaldehyde (MUC). While it has been shown that exposure to MUC reproduces many of the myelotoxic effects of benzene exposure in rodents (7), blood levels of MUC from benzene exposure have not been reported. Thus, while MUC has been show to have the potential to play a key role in benzene-induced toxicity, it's actual role, which depends on how much reaches the bone marrow during benzene exposures, is uncertain.

The second metabolite which has the potential to play a key role, and for which there are only very limited dosimetry data to date, is benzene oxide (15). In fact, not only is the dosimetry largely unknown, but the effects of benzene oxide on bone marrow have not been evaluated. Since benzene oxide is an epoxide, and is known to form hemoglobin adducts, now that it is know to achieve measurable blood levels one would suspect that it plays some role in benzene-induced toxicity. Thus, both the pharmacokinetics and the pharmacodynamics of benzene oxide should be examined.

There have also been some of studies on benzene dosimetry in humans (e.g., 16-18), but these have been restricted to analyses of changes in (expired) air concentration, blood levels of benzene itself, and urinary levels of metabolites, with the later being confounded by dietary levels of phenolics (19). So one of the largest unknowns is the blood levels of benzene metabolites in humans resulting from benzene exposure. It is theoretically possible to estimate these levels using physiologically-based pharmacokinetic (PBPK) modeling, given data now available on metabolism of benzene by human liver *in vitro*. But the existence of data which would allow such predictions to be checked would add considerable certainty to the process.

Another unknown is the potential presence of cytochrome P450 2E1 (CYP2E1) in human bone-marrow. CYP2E1 is found in rabbit bone marrow (20), but not that of the mouse (21). While the level of CYP2E1 is likely to be low in bone marrow compared to liver, the production of small amounts of reactive metabolites by CYP2E1 oxidation in the target tissue might have as much impact as the production of larger quantities of such metabolites in the liver, since in the later case much of the metabolites produced may not reach the bone marrow.

The final area where experimental confirmation is with regard to the theory of Medinsky and coworkers (22) that aspects of benzene and phenol metabolism can be explained by zonation of metabolic enzymes in the liver. Hedli *et al.* (23) reported the results of comparing benzene metabolism in the isolated, perfused mouse liver,

specifically comparing the results of normal (orthograde) vs. reversed (retrograde) perfusion. The results of Hedli et al are compatible with and support the zonation hypothesis, but this hypothesis predicts the primary effect to be on the metabolism of phenol, not benzene. In particular, since phenol is a substrate for sulfotransferases, which are concentrated in periportal regions of the liver, while CYPE1, which would oxidize phenol to hydroquinone, is concentrated more highly in centrilobular regions. Thus with normal perfusion of phenol one would expect the primary metabolite observed to phenol sulfate as compared to hydroquinone, while with reverse perfusion a much higher proportion of hydroquinone (and it's conjugates). Experiments to test this aspect of the hypothesis have not been performed, and would be a valuable contribution in supporting or refuting it.

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BENZENE-INDUCED GENOTOXICITY

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INTRODUCTION

Benzene (BZ) is a human leukemogen that also produces neoplasia at various sites in animal bioassays. Alternative explanations for BZ-induced neoplasia based upon genotoxic effects have been proposed in the literature. This study analyzed all available studies of DNA reactivity and genotoxicity reported in the literature for BZ and its metabolites, which involved 1400 individual test results. Based on the genotoxicity profile generated, alternative mechanisms were evaluated.

DNA REACTIVITY

Two types of studies that examined DNA reactivity have been reported in the literature: ^{32}P-postlabeling, which can directly detect and identify DNA adducts, and binding of radiolabeled BZ to isolated DNA. DNA binding following exposures to radiolabeled BZ has been found in both target and non-target tissues by *in vivo* experimental studies. In the tissues that exhibit clear neoplastic findings, no increased level of DNA binding is apparent, compared to organs that showed no neoplastic effects. Where adducts have been clearly detected by ^{32}P-postlabeling, these were not in apparent target organs for neoplasia in rodents. Conversely, in the tissues that exhibit clear neoplastic findings such as the Zymbal gland in rats, no DNA adducts have been reproducibly found. Additionally, DNA adducts have not been clearly identified by ^{32}P-postlabeling in either target or non-target tissues under dosage conditions that have provided evidence of neoplasia in experimental animals.

The postlabeled adduct levels were substantially lower (about 100-fold) than those determined by the DNA binding methods. The detection of binding by labeling methods are not necessarily an indication of covalent binding of chemical or its metabolite(s) to DNA since several non-DNA components could interfere in the interpretation of results. Also, these methods could detect adducts that do not have mutagenic potential. Consequently, the significance of DNA binding studies for neoplasia are not presently interpretable.

GENOTOXICITY STUDIES

In vivo, BZ was strongly and consistently positive for micronucleus formation. BZ itself was negative in the one, published, *in vitro* micronucleus assay, both with and without metabolic activation. In contrast, polyhydroxybenzene BZ metabolites, including phenol (PH), hydroquinone (HQ), catechol (CAT) and benzenetriol (BT), were positive *in vitro* in the micronucleus assay. Data for ring-opened BZ biotransformation products *trans,trans-*

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muconaldehyde; (*MUC) and *trans,trans*-muconic acid (MUC) were limited. Results for BZ in micronucleus assays were representative of those in other assays for cytogenicity. Exposure *in vivo* to BZ or *in vitro* to BZ biotransformation products caused structural chromosomes aberrations, chromosome loss (aneugenicity), and induced strand breakage. In these other *in vitro* cytogenicity assays, BZ and PH were negative or positive depending on the assay conditions, while HQ, CAT, BQ, and BT were consistently positive.

BZ *in vitro* gave mixed positive and negative results without activation in assays for aneugenicity and clastogenicity. The positive findings were primarily obtained in studies employing marrow cells or leukocytes. These cell types are known to contain various enzymes that are involved in BZ myelotoxicity and genotoxicity. In *in vitro* assays, the addition of induced rat liver microsomes did not have a consistently positive or negative effect on genotoxicity endpoints. This was attributed to various factors, including absence of enzymes critical to the true genotoxic mechanisms of BZ *in vivo* and, possibly, abundance of enzymes that could detoxify BZ biotransformation products.

No specific BZ metabolite has been identified to fully explain BZ genotoxicity. However, the polyhydroxylated metabolites and their semiquinone and quinone oxidation products are clearly involved. Some mixtures of BZ oxidative biotransformation products exhibited genotoxic synergy *in vitro*. Others were additive and others less than additive. HQ + PH + CAT or HQ + CAT were synergistic in micronucleus assays. This finding provides evidence that specific combinations of BZ biotransformation products may lead to much higher levels of genotoxicity than either product alone.

In contrast to the consistently positive data in cytogenicity assays, BZ and its biotransformation products, including MUC and *MUC, were negative in assays for reversion in *Salmonella*. Some positive results in assays for mammalian forward mutagenicity in culture were interpreted as reflecting cytogenetic mechanisms, in the absence of sequence or other information necessary to conclusively determine the nature of the underlying event. The mammalian forward mutagenicity findings are consistent with a cytogenetic agent that causes chromosome breakage, loss, and/or rearrangement.

MECHANISMS OF GENOTOXICITY

Four specific mechanisms have been most frequently cited in the literature to explain BZ genotoxicity, as follows: 1) DNA-reactive BZ metabolites forming adducts or cross-links; 2) Oxidative DNA damage leading to strand breaks and missense mutations; 3) Clastogenesis due to topoisomerase II inhibition; 4) Aneugenesis due to damage to components of the mitotic apparatus. The genotoxicity profile for BZ compared to that of other agents known to produce genotoxicity by specific mechanisms provided a means to differentiate between the possible contributions of any or all of these mechanisms to the BZ genotoxicity findings.

Polyhydroxylated BZ metabolites have been shown to inhibit topoisomerase II, which produces a double strand break allowing the passage of a second double helix through the break. The genotoxicity profile of BZ matches that of a topoisomerase II inhibitor, which is capable of producing all of the positive genotoxicity results through a mechanism involving a stable BZ-topoisomerase II-DNA complex. The result of forming this complex is sister chromosome exchange, non-homologous recombination, gene deletion and rearrangements. The genotoxicity profiles do not support DNA-reactivity, generation of reactive oxygen species, spindle poisoning, or ribonucleotide reductase inhibition as the underlying mechanism.

HUMAN LEUKEMOGENESIS

Studies of the genotoxicity of BZ and its metabolites in humans and in cell derived from humans *in vitro* are consistent with the genotoxicity data in animals, animal-derived cells *in vitro*, and bacterial systems. A mechanism involving DNA reactivity is not supported by the lack of identified DNA adducts under relevant exposure conditions. Furthermore, the lack of mutagenesis in bacterial tests by BZ metabolites also supports a lack of DNA reactivity.

The production of BZ metabolites in humans is qualitatively similar to rodents. Therefore, inhibition of topoisomerase II also appears to be the best explanation of the genotoxicity results in humans for BZ and its metabolites. Topoisomerase II inhibitors are known to produce leukemia in humans although the some of the chromosomal alterations are different from those involved in BZ-induced leukemia. Consequently, if the results of genotoxicity from human studies are relevant to the mode of action for BZ-induced leukemogenesis, inhibition of topoisomerase II is the most likely mechanism.

Benzene-induced Genotoxicity: A Different Perspective

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Although benzene is recognized as a human carcinogen, the critical mechanisms underlying its genotoxic and leukemogenic effects remain elusive. A large number of *in vitro* and *in vivo* genotoxicity studies of benzene and its metabolites have been conducted. However, given the requirement for metabolic activation, the complexity of benzene's metabolic pathways, and the potential for interactive effects, *in vitro* studies of benzene genotoxicity are currently of limited usefulness. Recently, a number of *in vivo* studies have been conducted using both conventional and recently developed molecular techniques which are providing valuable insights into the types of genetic alterations associated with exposure to high levels of benzene. The results of these animal and worker studies are largely consistent with earlier investigations employing more traditional approaches. These studies have shown that benzene is weakly effective in inducing specific gene mutations and binds inefficiently to bone marrow DNA. In contrast, similar studies have shown that benzene is effective in inducing large-scale chromosomal alterations. Chromosome breakage represents probably the most common genetic lesion induced by benzene but other structural aberrations such as translocations can also be formed. Aneuploidy, a type of numerical chromosome aberration, is modestly increased with benzene exposure. This pattern of genotoxicity is consistent with that exhibited by other known human leukemia-inducing agents which have consistently been shown to induce chromosomal alterations as well as bone marrow toxicity. In addition, a recent update on two cohorts being followed by the European Study Group on Cytogenetic Biomarkers and Health (Hagmar et al., Cancer Res. 58:4117-21, 1998) has shown that individuals exhibiting elevated frequencies of chromosomal aberrations are at significantly elevated risk for developing cancer and that this risk increases with time. Similar types of clonal structural and numerical aberrations are seen in human leukemias and appear to play a critical role in leukemogenesis. This convergence of evidence indicates that the induction of chromosomal alterations by benzene is likely to play a key role in its genotoxic and leukemogenic effects. Although recent studies are making progress in identifying genotoxic mechanisms, much of this work is in its early stages and needs to be independently replicated. Identification of the metabolites contributing to benzene's genotoxic effects, the mechanisms underlying the alterations, and the specific chromosomes and chromosomal regions involved should be an important focus for future research.

Benzene Leukemogenesis: An Environmental Carcinogen Induced Tissue Specific Model of Neoplasia using Genetically Altered Mouse Models:

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Benzene is carcinogenic to laboratory rodents as well as to humans. However, the mechanism(s) of benzene toxicity and carcinogenicity are not well understood and there are no highly reproducible experimental rodent models for investigation that are not confounded by viral leukemogenesis. A major objective of our laboratory is to develop carcinogen induced tissue specific models of neoplasia. Critical to both prospective identification of carcinogens and investigation of carcinogenic mechanism(s) is the induction of tumors within a sufficiently short time frame such that sporadic tumors will not confound investigation or interpretation due to aging or attendant strain specific tumors. Ideally, tumor induction should be observed at tissue/organ sites consistent with transspecies carcinogens (i.e., concordant at common sites across species). In addition, a mode or a mechanistic basis, such as loss or mutations of a tumor suppressor gene or oncogene, for tumor induction should be easily and rapidly identified for use in risk assessment. Finally, to increase confidence in the extrapolation of data from rodent to human, the mode or mechanism must be consistent with our current knowledge of human. Genetically altered mouse models, which have defined genetic lesions, which are critical but insufficient alone to induce cancer, are potential models for investigation of chemical carcinogenesis and risk assessment.

We are currently investigating benzene toxicity and carcinogenicity in different genetically altered mouse models: 1) FVB/N-Tg.AC (v-Ha-*ras*) mouse in which the transgene is inducible by both mutagenic and nonmutagenic carcinogens, 2) the C57BL/6- or FVB/N-Trp53 mouse heterozygous for an inactivated p53 allele, and 3) the FVB/N-Trp53 heterozygous mouse hemizygous for the Tg.AC (v-Ha-*ras*) transgene. Benzene (>99.9% pure) was administered topically (0 or 200 µl neat; 2×/da; 2×/wk for 14-26 weeks) or orally (intubation) (0 – 600 mg/kg/BW; 5×/wk) for 26 weeks starting at 8 to 12 weeks of age to the selected lines plus coisogenic wildtype control mice.

Oral administration of benzene to C57BL/6-Trp53 heterozygous mice resulted in the induction of sarcomas of the subcutis (16/39), thymic lymphoma (3/39), and a pancreatic carcinoma (1/30). No tumors were observed in the 20 vehicle treated controls. The frequent loss of the remaining wildtype p53 allele (LOH) was observed (13/16 sarcomas; 3/3 thymic lymphomas; 1/1 pancreatic acinar carcinoma). Oral administration of benzene to FVB/N-Tg.AC (v-Ha-*ras*) mice resulted in a dose related mortality and bone marrow toxicity but no tumors. In three independent studies, topical application of benzene resulted in a dose-related induction of neoplasia (papillomas and spindle cell tumors) and granulocytic leukemia. Results from topical application of benzene to bigenic mice are currently under review.

We conclude from these studies that there are differences in benzene toxicity and potential carcinogenicity depending upon the route of administration and the selection of the test species strain. Furthermore, benzene activation of PKC and other signaling events lead to genetic events and resulted in skin and hematopoietic malignancies under these experimental conditions. These genetically altered mouse models may be suitable for dose metric and mechanistic studies of benzene leukemogenesis by the inhalation route of exposure.

Myeloid Leukemia After Cytotoxic Therapy and Other Hematotoxins.

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This work was supported by grants PO1 CA-40046 and CA-14599 from the National Institutes of Health.

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BP-00011582

Therapy-related leukemia is a neoplastic hematopoietic disorder arising in most cases from a multipotent stem cell and in a few cases from a lineage-committed progenitor. Therapy-related acute myeloid leukemia (t-AML) describes a clinical syndrome that exhibits important differences from AML that arises de novo (1). The terms "therapy-related" or "treatment-related" leukemia are descriptive and are based on a patient's history of exposure to cytotoxic agents. They imply a causal relationship, but the mechanism of this remains to be established. These terms may ultimately be too restrictive, since the leukemia that develops after exposure to benzene or atomic bomb irradiation is very similar or identical to the therapy-related leukemia syndrome. In the future, as various subtypes of leukemia are distinguished by specific genetic alterations, the terms de novo (or primary) and therapy-related leukemia will likely be discarded and specific etiologies incorporated into the diagnostic nomenclature.

The development of therapy-related second neoplasms provides a unique, ethically acceptable environment for studying the effects of mutagens on carcinogenesis in man. The long latency period characteristic of therapy-related cancers after initial mutagenic exposure suggests that "two hits" or perhaps multiple intermediate steps are required for full expression of the malignant phenotype. It has not been possible to determine whether the development of a therapy-related neoplasm is a stochastic event, or whether certain individuals are at higher risk (perhaps due to a DNA-repair deficiency or a heritable predisposition) and thus might be identifiable in advance (2).

In general, two paths of investigation have been explored. The first involves meticulous clinico-pathological and cytogenetic analysis of individual cases as they present with therapy-related leukemia (3). The second involves large scale epidemiological surveys of patients at risk. There are now many such studies of each type in the literature. Patients with Hodgkin's disease were the first large cohort of relatively uniformly treated patients who experienced prolonged survival. After long-term follow-up, hundreds of cases of therapy-related leukemia have now been reported on and analyzed. It soon became clear that the risk of therapy-related leukemia was shared by

patients with other cancers and even non-malignant disorders if they had received cytotoxic treatment (4).

CLASSICAL THERAPY-RELATED LEUKEMIA

In the classic form of therapy-related leukemia that follows treatment with alkylating agents and/or radiation therapy, the blood and bone marrow findings resemble those seen in the primary myelodysplastic syndromes (MDS). Anemia and thrombocytopenia are extremely common. Leukopenia may also be present. Marked dysplastic changes are observed in all three cell lines. Early in the course of disease, red cell poikilocytosis may be particularly notable in the peripheral blood film. The bone marrow may have variable cellularity, but is most often hypercellular. Hypocellular and even aplastic marrows are seen in some cases. Mild to marked reticulin fibrosis may be present. The degree of dysgranulopoiesis and dysmegakaryocytopoiesis is typically greater than that observed in primary MDS.

About two-thirds of patients present with fewer than 30% blast cells in the marrow and <5% blasts in the blood, and therefore, these patients have been diagnosed as t-MDS. However, unlike primary MDS which may have a long preleukemic phase, patients with therapy-related leukemia have a more prominent arrest of hematopoietic maturation and a more rapid accumulation of blasts. Typically, the t-MDS phase lasts for about six months. As these cases evolve to more overt leukemia, features characteristic of FAB subtypes M1, M2, or M4 are most common. However, there are difficulties in classifying therapy-related leukemia according to the FAB criteria designed for AML de novo since most cases demonstrate trilineage involvement and often overlap several subtypes. Auer rods are rarely seen, and myeloperoxidase and non-specific esterase reactivity are often only weakly expressed. There is a continuum of clonal expansion and dedifferentiation that occurs in the neoplastic clone and subclones. Shortened survival is more a function of failure of normal hematopoiesis rather than rapid accumulation of bone marrow blast cells.

Clonal chromosomal abnormalities, often of a complex nature, are identified in most cases of classical therapy-related leukemia (1-4). Loss of part or all of chromosomes 5 and/or 7 are the characteristic findings, and have been reported in overt 90% of cases in some series (3). Table 1 shows the distribution of cytogenetic abnormalities observed in 270 patients with therapy-related leukemia studied at the University of Chicago. The karyotypes are often complex. The most common single abnormality is monosomy 7, followed in frequency by deletion of the long arm of chromosome 5 [del(5q)] and by monosomy 5. These same abnormalities are observed in primary MDS and AML de novo, especially in older patients and those with occupational exposure to potential carcinogens. Their frequency, however, is clearly higher in therapy-related leukemia. Recent molecular investigation has focused on identifying a putative leukemia suppressor gene in chromosome band 5q31, a critical region that is consistently deleted in leukemia cells with 5q abnormalities.

LEUKEMIA FOLLOWING TOPOISOMERASE II INHIBITORS

Whereas classic t-AML is characterized by abnormalities involving the long arms of chromosomes 5 and/or 7, the leukemias secondary to agents that target topoisomerase II result in translocations involving the MLL gene on chromosome 11, band q23, and less commonly, the AML1 gene on chromosome 21, band q22 (5). At first, the association was linked only to the epipodophyllotoxins, etoposide and teniposide. However, subsequent reports have also implicated DNA intercalating agents such as doxorubicin. In contrast to classic t-AML, these leukemias have a much shorter latency between initiation of chemotherapy for the primary cancer and the development of leukemia (Table 2). In addition, a preceding myelodysplastic syndrome is not associated with these leukemias. The t(11q23) cases primarily have monoblastic (M5) or myelomonocytic (M4) phenotypes, but cases of AML-M1 and M2 as well as ALL have been described. The t(21q22) cases are typically AML M2.

DIFFERENT GENETIC MECHANISMS FOR LEUKEMOGENESIS

The particular mechanisms of DNA damage that lead either to chromosomal deletions or to balanced translocations may underlie the differences in latencies between the two forms of therapy-related leukemia (5). In the case of chromosomal deletions, one allele of a putative tumor suppressor gene may be inactivated. Before the affected cell would gain a proliferative advantage, however, the second allele would also have to be deleted or mutated. Additionally, losses of both alleles of an individual tumor suppressor may not be sufficient to confer a malignant phenotype. As described in the model of colorectal tumorigenesis, multiple tumor suppressor genes or oncogenes may need to be mutated to ultimately transform a cell. This series of genetic changes may require an extended period of time, thus explaining the long latency of alkylator-induced t-AML. In contrast, balanced chromosome translocations result in the activation of cellular oncogenes in a dominant fashion. These rearrangements, such as those involving the MLL gene at 11q23, may yield a fusion gene that acts as a dominant oncogene. Whereas this fusion gene alone may not be sufficient to transform an hematopoietic progenitor cell, relatively fewer genetic events may be required to proceed to the leukemic phenotype. In line with this hypothesis, 70-80% of all acute leukemias that occur de novo in infants, both lymphoid and myeloid, involve the MLL gene. Moreover, these cases have even been reported in the neonatal period. Thus, 11q23 translocations can clearly induce the formation of leukemia over a short interval of time. The striking incidence of MLL gene rearrangements in infant leukemias suggests a potential genetic susceptibility to translocations at this locus. If this were the case, perhaps only certain patients with an as yet unknown DNA repair deficiency might be susceptible to the mutagenic effect of topoisomerase II inhibitors.

As the numbers of cancer survivors increase after conventional cytotoxic treatment, the incidence of therapy-related leukemia will undoubtedly rise. As further understanding about mechanisms of mutagenesis develops, it is likely that certain individuals who have increased susceptibility to the leukemogenic activity of particular agents can be identified.

Individuals demonstrating benzene-associated hematotoxicity should undergo careful evaluation by clinical, morphological, cytogenetic, and molecular techniques in order to discover the intermediate steps in the progression to leukemia.

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Table 1. Cytogenetic Abnormalities in Therapy-Related Myeloid Leukemia (University of Chicago series)

No. of patients	270
No. with clonal abnormalities	252 (93%)
Abnormalities of chromosomes 5 and/or 7	191 (71%)
Chromosome 5 only 54 (20%)	
Chromosome 7 only 76 (28%)	
Chromosomes 5 and 7 61 (23%)	
t(11q23)	9 (3%)
t(3;21) or t(8;21) or t(21q22)	8 (3%)
t(15;17)	4 (1%)
+8	7 (3%)
inv(16)	4 (1%)
-13 or del(13q)	3 (1%)
Other clonal abnormalities	26 (10%)

Data from MM LeBeau, 1998

Table 4. Contrasting Features of Therapy-Related Leukemia Secondary to Either Alkylating Agents or Topoisomerase II Inhibitors

	Chromosome Abnormality	Preleukemia Phase	FAB Morphology	Latency	Response to Induction Chemotherapy	Long-term Survival	Chemotherapy Drugs
Alkylating Agents	del(5q), -5 del(7q), -7	MDS	Not classifiable by current criteria	5-7 years	poor	poor	melphalan, mechlorethamine, chlorambucil, cyclophsophamide, carmustine, lomustine semustine procarbazine, dacarbazine, mitolactol
Topoisomerase II inhibitor	11q23 translocations 21q22 translocations	none	Usually M4, M5; some M1, M2 and ALL-L1	6 months-5 years	good	poor	etoposide, teniposide, actinomycin D, doxorubicin, 4 epi-doxorubicin mitoxantrone
Various agents	t(15;17)	none	M3	2-3 years	good	good	bimolane
	inv(16)	none	M4Eo	<3 years	good	good	

BP-00011589

THE INITIAL TRANSFORMING EVENT IN MYELODYSPLASTIC SYNDROMES MAY BE VIRAL

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SUMMARY:

The myelodysplastic syndromes (MDS) are indolent clonal disorders which predominate in the elderly and present with the clinical paradox of variable cytopenias despite generally cellular bone marrows (BM) (1, 2). A novel paradigm has recently been proposed to explain the variegated nature of the pathology encountered in MDS (3). An as yet poorly defined event(s) confers a growth advantage on a pluripotential BM stem cell whose proliferation eventually overwhelms the marrow leading to monoclonal hemopoiesis. In a certain percentage of MDS patients, dual acting cytokines from unexplained sources further confound this picture by providing a stimulus to the early precursors to divide and inducing apoptotic death of their maturing progeny. It is important to note that apoptosis is found in all stages of the cell cycle, particularly in cells actively synthesizing DNA (3). This situation of apparent "antonymy" where a cell is found to be simultaneously in S-phase and apoptotic appears unique to MDS and could not be documented in a variety of other cancers such as head and neck, breast, ovarian, brain, lymphomas and de novo acute leukemias (3). Anyhow, this excessive proliferation and apoptosis results in the clinical syndrome of pancytopenia (excessive intramedullary apoptosis of maturing cells) in the setting of hypercellular marrows (rapid proliferation of the transformed progenitors). The cytokines responsible for the genesis of apoptotic death of hematopoietic cells include tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-ß) and interleukin-1 beta (IL1-ß) (4, 5). In some MDS patients, the growth advantage conferred upon the stem cell may be matched by a propensity to undergo premature apoptosis as the cells differentiate. In still other patients, the genesis of cytopenias may not be excessive intramedullary apoptosis at all, but may be excessive retention of hematopoietic cells in the BM due to dysregulation of adhesion molecules and/or their receptors.

What can be said with certainty is that hematopoiesis in MDS is monoclonal where both lymphoid and myeloid cells appear to be the progeny of the same parent (6-7). There is however, no incontrovertible proof that the "initial transforming event" (or series of events) occurs in a pluripotential BM stem cell in MDS. Rather, the diseased nature of the parent stem cell is inferred from the abnormalities manifest in its daughters, the most prominent being dysplastic maturation and the presence of monoclonal hemopoiesis. In fact, the reason why MDS is considered as representing a malignant state is because of its clonal nature and presence of karyotypic abnormalities which frequently undergo evolution as the disease progresses. Monoclonality in MDS should not be confused with monoclonality encountered in other malignant states such as acute myeloid leukemia (AML). In the case of AML, the leukemia cells are clearly descended from a single transformed parent, however, residual hematopoiesis is polyclonal (8). In MDS on the other hand, cells belonging to all three lineages (erythroid, myeloid, megakaryocytic) as well as probably the B -

lymphocytes are monoclonal or descended from the single transformed parent cell (6). MDS resembles the chronic phase of chronic myeloid leukemia (CML) where the normally differentiated (as opposed to dysplastic mature cells in MDS) erythroid, myeloid and megakaryocytic cells all contain the Philadelphia (Ph^1) chromosome. The transforming event in CML appears to be the bcr-abl genetic abnormality resulting from the Ph^1 translocation since the BM returns to a polyclonal state upon regression of this cytogenetic anomaly following interferon therapy (9). In MDS however, even the frequently encountered cytogenetic abnormalities such as those affecting chromosomes 5 and 7 may be epiphenomena or secondary events since they often appear to be present in only a subpopulation of cells in a marrow which is otherwise monoclonal. In summary therefore, the earliest consequence of the initial transforming event in MDS is monoclonal hemopoiesis while cytogenetic abnormalities represent disease evolution. In CML on the other hand, absence of monoclonal hemopoiesis upon eradication of the Ph^1 chromosome appears to implicate the bcr-abl abnormality as the "transforming event".

What Is The "Initial Event" In MDS?

Since there are certain karyotypic anomalies which occur with a regular frequency in MDS, it is not unreasonable to look for the "initial transforming event" in genic changes such as loss of a tumor suppressor gene or constitutive activity of a proto-oncogene associated with the most commonly involved areas of the affected chromosomes. However, even if such a gene was identified, the question of what caused its loss or dysfunction would still remain obscure. In this paper we would like to propose that MDS can begin as a viral disease. We further propose that the virus may infect a BM stem cell, a BM stromal cell or a cell belonging to the immune system. Abnormal cytokine expression as a consequence of the infection could change the bone marrow microenvironment in such a way that only a rapidly proliferating stem cell would survive in this new setting, thus accounting for the monoclonal hemopoiesis seen in MDS. The daughters of such a rapidly proliferating abnormal stem cell might also undergoes premature apoptosis either as a result of the adverse microenvironment or as a direct consequence of increased divisions in the parent since it has been demonstrated that less than 10% of $CD34^+$ cells dividing once in vitro were apoptotic as compared to 25% of $CD34^+$ cells which had divided 4 or more times (10). Taken together, these abnormalities would produce the syndrome of monoclonal hemopoiesis, pancytopenia and hypercellular marrows.

If this hypothesis is correct then the first essential question pertains to the identity of the virus. We propose that the virus is probably a common human pathogen that has developed long-term residence in the host without causing clinically significant ongoing disease. The virus however is capable of reactivation from its latency phase and even be made "oncogenic" in the presence of the right

"promoting conditions". These promoting events would include immunosuppression as a consequence of aging, bone marrow transplant or other chemo-toxic exposures, a second viral infection (acquired immune deficiency syndrome or AIDS) or finally any other infectious and/or non-infectious conditions which lead to upregulation of pro-inflammatory cytokines. It is also worthwhile to remember that prolonged, chronic exposure to the promoting events probably carries far more oncogenic potential than short-lived surges of cytokines or exposure to cytotoxic agents.

On the whole, it is important to emphasize that in the majority of patients with MDS, along with abnormalities of the BM stem cell, there also appears to be a dysregulation of the cytokine milieu in an otherwise exquisitely balanced and finely-tuned BM microenvironment. In fact, if we suppose that the "initial event" in MDS is related to the BM microenvironment and dysregulated cytokines, then it is highly probable that the altered landscape would profoundly affect the fitness of the organisms that normally reside therein. For example, mutated progenitor cells such as those bearing the bcr-abl translocation or t(14, 18) abnormalities have been clearly demonstrated in healthy individuals (11, 12), but probably remain at a low frequency because "normal" BM microenvironmental conditions are not conducive towards their expansion. In the changed environment however, these mutated or otherwise abnormal clones can conceivably acquire a growth advantage over their normal counterparts. This would eventually lead to monoclonal hemopoiesis, with a rapidly proliferating transformed parent whose daughters die prematurely either due to the action of excessive pro-inflammatory cytokines or as a consequence of increased divisions, or both. The syndrome of pancytopenia, hypercellular marrow, monoclonal hemopoiesis with excessive proliferation/apoptosis and abnormal cytokines expression would ensue. This situation could persist as such with increasing deterioration of the existing clinical problems such as worsening cytopenias. On the other hand, acquisition of a mutation in one of the daughters of the transformed parent could lead to loss of its ability to apoptose and mature, thereby giving rise to a new population of blastic cells and the transformation of a simple refractory anemia (RA) to refractory anemia with excess blasts (RAEB). Progression to acute myeloid leukemia (AML) would depend on the rapidity with which these blast cells would be able to undergo substantial clonal expansion. Monoclonal hemopoiesis would clearly predispose to such accumulation of mutations in a daughter cell. During all of this disease causation and evolution, maintenance of the abnormal cytokines milieu in the BM microenvironment would be essential for disease persistence. A second possibility is obviously related to transformation of a hematopoietic stem cell which would rapidly divide/apoptose and give rise to abnormal cytokine production eventually leading to the same scenario described above. A virus could accomplish either of these possibilities by infecting an early BM progenitor cell or a BM stromal cell and we propose that a DNA virus belonging to the herpes virus family could be one such pathogen.

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ROLE OF NITRIC OXIDE IN BENZENE INDUCED HEMATOSUPPRESSION

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It is becoming increasingly apparent that nitric oxide plays a multifunctional role in regulating inflammatory processes in the body. Although nitric oxide and its oxidation products are cytotoxic toward certain pathogens, they can also cause tissue injury, suppress proliferation and induce apoptosis. Cytokines and growth factors released at sites of inflammation or injury stimulate both immune and nonimmune cells to produce nitric oxide. No where in the body is this more detrimental than in the bone marrow since the continuous production of hematopoietic precursors is essential for normal blood cell maturation. Our laboratories have discovered that, in response to inflammatory mediators such as bacterial derived lipopolysaccharide (LPS), interferon-γ (IFN γ), and tumor necrosis factor-α (TNF α), bone marrow leukocytes, consisting predominantly of macrophages and granulocytes readily produce nitric oxide. This response is dependent on l-arginine and blocked by the nitric oxide synthase inhibitors, monomethyl-l-arginine (L-NMA) and aminoguanidine. Western and northern blot analysis have revealed that bone marrow cell nitric oxide production is mediated by the actions of an inducible form of the enzyme, nitric oxide synthase (NOS2). Additional studies have demonstrated that nitric oxide production by bone marrow leukocytes is augmented by hematopoietic growth factors including interleukin-3, macrophage colony stimulating factor, and granulocyte-macrophage colony stimulating factor. Production of nitric oxide by bone marrow leukocytes is associated with impaired hematopoiesis, a response that is potentiated by colony-stimulating factors. Treatment of mice with benzene, which suppresses bone marrow cell development, markedly enhances the ability of bone marrow cells to produce nitric oxide in response to inflammatory mediators alone and in combination with hematopoietic growth factors. Moreover, mice deficient in NOS2 as well as TNFα are protected form benzene induced bone marrow depression. Taken together, these data suggest that nitric oxide may be an important mediators of benzene-induced bone marrow suppression (Supported by NIH grants ES06897 and ES05022 and the Burroughs Wellcome Fund).

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Molecular Models of Benzene Leukemogenesis. Richard D. Irons, Molecular Toxicology and Environmental Health Sciences Program, Dept. of Pharmaceutical Sciences, School of Pharmacy; Dept. of Pathology, School of Medicine; and Comprehensive Cancer Center, University of Colorado Health Sciences Ctr., Denver, CO., USA.

Several years ago, we proposed a multistep model for the evolution of myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) developing secondary to drug or chemical exposure. The model described a multistep progression in which a relatively small number of alternative pathways converge in the development of leukemia. Its essential elements were as follows: Chemical-induced transient changes in cytokine response result in altered regulation of proliferation in the hematopoietic target cell compartment; Increased proliferation predisposes to development of an early clonal chromosomal abnormality, primarily involving deletion of all or significant regions of individual chromosomes, most often involving chromosomes 5 and 7; Interaction of the emerging clone with the hematopoietic stromal environment leads to clonal selection and outgrowth; Additional random mutational events occurring in dividing cells predispose to further selection resulting in the ultimate development of the leukemic phenotype.

We have found the model to be a useful tool for use in integrating hematopoietic paradigms for the origin and progression of MDS/AML with expanding information on the patterns and molecular events associated with secondary AML. Since its inception, descriptive, mechanistic and clonal data has emerged to both reinforce and amplify various aspects of the model architecture, as well as to pose additional questions with respect to the specific applicability of the model to benzene leukemogenesis.

This presentation will outline and review the model in light of the current state of knowledge with respect to its applicability to benzene. Mechnanistic and cytogenetic findings from our laboratory and others will be discussed

with respect to their impact on the model and in assessing its relevance to benzene. Finally, these results will be evaluated with a view toward identifying the types of information that would be most useful in further refinement and validation of a benzene leukemia model as well as in critically evaluating endpoints of interest as potential biomarkers of benzene exposure.

Utility of a Mouse Model for Studying the Effects of Benzene on the Myeloid Lineage. George F. Kalf, Ph.D. Department of Biochemistry and Molecular Pharmacology and the Kimmel Cancer Institute, Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA.

It was previously demonstrated in this laboratory (1) that the acute administration to mice of benzene or hydroquinone (HQ), a metabolite found in the bone marrow, causes a severe depression in the number of cells of the bone marrow except those of the granulocytic lineage which not only survive, but differentiate and increase in number. Renz and Kalf (2) provided an explanation for this observation by the demonstration that in mice benzene in vivo or HQ in vitro caused an inhibition of the production of interleukin-1 by stromal macrophages. Interleukin-1 is obligatory for the induction of cytokine production by stromal fibroblasts without which the progenitor cells in the bone marrow do not survive.

These results suggested that the granulocytic progenitor cells survive and differentiate because HQ can substitute for granulocyte-colony stimulating factor (G-CSF), the requisite cytokine for granulopoiesis, and induce differentiation in the myeloid stem cell. The ability to alter cytokine-dependent growth and differentiation in hematopoietic progenitor cells appears to be a property of agents that have a potential for causing secondary leukemia in humans. It was important to know whether HQ had a deleterious effect on cytokine-induced proliferation and/or differentiation of the restricted myeloid stem cell, the myeloblast, and if so, whether HQ could effect any of the changes typically seen in the phenotype of leukemia cells in culture.

We utilized the continuous IL-3-dependent mouse cell line, 32D clone 3(G) as our model system (1,4). This cell line was derived by Greenberger et al. (3) from normal bone marrow of C3H/HeJ mice. It has many features that allowed us to determine whether HQ was affecting myeloid differentiation by interacting with essential cytokines. The cell line is: 1) myeloblastic and has a normal karyotype; 2) nonleukemic as indicated by its inability to cause tumors in nude mice or to generate cytokine-independent clones; 3) IL-3-dependent for survival and proliferation; and 4) is induced to undergo terminal granulocytic differentiation in the presence of the physiological inducer of the granulocytic lineage, G-CSF.

Myeloblasts were cultured in the presence of a concentration of IL-3 low enough to sustain survival and a level of proliferation that would not overly compete with the induction of differentiation by G-GSF or HQ (1,4). Morphological assessment of granulocytic differentiation was performed on Cytospin-prepared, May-Grünwald/Giemsa-stained cell monolayers plated on polylysine-coated glass slides. Differentiation was expressed as the percentage of total cells showing some stage of granulocytic morphology. Total differentiation was defined as the combined percentages of promyelocytes, myelocytes, metamyelocytes and banded/segmented, or terminally differentiated cells, and

was determined by averaging the percentages of total differentiation out of 200 cells on each of triplicate slides. Terminal differentiation was assessed by counting only segmented granulocytes. The induction of terminal granulocytic differentiation was verified by demonstrating that G-CSF or HQ caused the induction of various functional properties of granulocytes such as superoxoxide production (measured as nitroblue tetrazolium (NBT) reduction), the expression of specific enzymes (i.e. chloroacetate esterase), and the appearance of granulocytic-specific cell surface antigens. No monocytes were induced. IL-3, while sustaining survival and proliferation of the myeloblasts, did not induce differentiation.

HQ (1-2 μM) was shown to induce total granulocytic differentiation in comparison with G-CSF as assessed by morphology and NBT reduction (1,4). The putative inducing agent appears to be benzoquinone (BQ) from the myeloperoxidase-mediated oxidation of HQ in the myeloblast since: 1) resorcinol, a non oxidizable structural analog of BQ that does not serve as a substrate for myeloperoxide, does not induce differentiation, and; 2) the concomitant presence of indomethacin, a peroxidase inhibitor, with HQ inhibits HQ-induced differentiation.

G-CSF effects the initiation of differentiation in the myeloblast by the upregulation of the 5-lipoxygenase (LPO) pathway for the production of leukotriene D_4. an essential intracellular mediator of G-CSF transmembrane signaling. With the use of highly specific LPO inhibitors it was determined that BQ did not affect the LPO pathway for the production of LTD_4 in the myeloblast (4). Because of the ability of BQ to interact covalently with proteins containing sulfhydryl groups, it was anticipated that BQ might induce granulocytic differentiation in myeloblasts by activating the LTD_4 receptor thus obviating the requirement for LTD_4. Concomitant addition of the specific receptor antagonist, MK-571, with HQ showed a concentration-dependent inhibition of induction of differentiation by both HQ and G-CSF.

LTD_4 was shown to replace G-CSF in a concentration-dependent (10^{-9} to 10^{-5} M) induction of granulocytic differentiation. HQ showed a qualitatively similar concentration-dependent $5X10^{-7}$ to $4X10^{-6}$ M) induction of differentiation allowing the comparison of the nature of the differentiation induced by the binding of the two ligands to the receptor. Competition studies involving the addition of various concentrations of receptor antagonist either prior to or after treatment of the cells with HQ or LTD_4 suggest that BQ binds more tightly to the receptor than the specific antagonist.

G-CSF, LTD_4 and HQ each induced about 97% total differentiation. Terminal differentiation as a percentage of the total is about 50% in the case of G-CSF and LTD_4, whereas HQ only induced about 15 % terminal differentiation. The fact that HQ and LTD_4 both putatively work through the same receptor, but cause significantly different levels of terminal differentiation led us to focus on a comparison of the consequence of the interaction of the two ligands with the receptor. We carried out a morphological analysis of the kinetics of stage-specific granulocytic differentiation over a 6-day period induced by HQ in comparison with LTD_4. LTD_4–induced differentiation showed a predominance of mature,

segmented granulocytes at Days 2 through 6, whereas, HQ-treated myeloblasts showed an incomplete differentiation profile at each day studied that was arrested at the myelocyte stage and showed very few mature granulocytes.

Receptor antagonist (10^{-8} M) was capable of competing effectively with G-CSF or LTD_4 for the receptor as measured by total differentiation induced, however, it could not compete with HQ suggesting that BQ may be binding covalently to the receptor and thus constitutively activating it. HQ in the presence of G-CSF or LTD_4 caused a shift in the differentiation profile from one of terminal granulocytes to one of immature myelocytes. This result suggests that in the bone marrow, where HQ and G-CSF may be present at the same time, the pattern of terminal granulocytic differentiation will be shifted to that of predominately an incomplete differentiation to myelocytes that retain proliferative capacity.

If HQ, at concentrations that induce granulocytic differentiation, is added to a culture of myeloblasts growing in the presence of IL-3, a 2 to 3-fold increase in the number of myeloblasts is seen (5). We have determined that this increase does not result from an ability of HQ to deliver a proliferative signal to the myeloblast or from its ability to synergize with IL-3 to increase its proliferative signal. In the absence of IL-3, HQ did not stimulate cell growth, but did delay the death of IL-3-deprived myeloblasts. If IL-3 is withdrawn from the myeloblast, 90% of the cell population undergoes programmed cell death within 48 hours as determined by the observation of morphological changes such as cytoplasmic condensation, cell volume reduction, vacuolization, densely staining micronuclei, apoptotic bodies, and the presence of internucleosomal DNA fragments observed as a DNA ladder in a gel. The relevant question became whether the increase in the number of myelocytes that occurs when HQ induces differentiation can be attributed to the proliferative capacity of the myelocyte or to an ability of HQ to prevent or delay apoptosis. HQ at concentrations (1-2μM) that induce differentiation in myeloblasts were shown to prevent apoptosis induced by either IL-3 withdrawal or treatment with staurosporine (5) We have previously shown (6) that BQ inhibits the activity of IL-1 converting enzyme (ICE), a member of a family of caspace proteases involved in the final steps in the regulation of apoptosis. In the myeloid lineage, the caspace involved is caspase 3. BQ (2μM) was shown to inhibit recombinant caspace 3 by 50 per cent in a kinetic assay involving a specific fluorescent peptide containing the specific active site sequence of the caspace family of proteases. These results suggest that HQ via conversion to BQ prevents apoptosis in a proliferating population of early myeloid progenitors.

In summary: 1) Benzene and HQ induce an altered pattern of granulopoiesis in the mouse; 2) In a model culture system, HQ by binding to and constitutively activating the normal signal-driven LTD_4 receptor, induces an incomplete differentiation pattern in myeloblasts where the progenitor cell is the proliferating myelocyte; 3) HQ can compete with the physiological inducer, G-CSF, in the induction of terminal granulocytic differentiation in the myeloblast such that the differentiation profile is changed from one of mature segmented granulocytes to that of immature myelocytes; 4) HQ, by inactivation of an

essential caspace can prevent programmed death in myelocytes. Taken together, these results indicate that, in the presence of HQ in culture or in the bone marrow, myeloblasts can be induced to differentiate into an expanding clone of early progenitors that lack the capacity for terminal differentiation and thus have two phenotypic properties of leukemic cells in culture.

The use of mouse myeloblasts as a model system provides an ability to study epigenetic changes resulting from exposure of myeloblasts to various metabolites of benzene and to compare the results with those obtained in the benzene-treated mouse mouse or alternatively to verify those obtained with myeloblasts by testing in the mouse.

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Benzene and Lymphohematopoietic Malignancies in China.

Richard B. Hayes, PhD

Cancer risk was assessed in a large cohort of benzene-exposed workers in China(1-3). Translational studies were also carried out among workers with current exposure to benzene (4) and among workers with a history of benzene poisoning (5), to provide insight into benzene carcinogenesis in humans.

Cohort study of benzene-exposed workers. The cohort of benzene exposed workers was comprised of men and women employed between 1972-87 in 1,427 selected benzene-exposed work units (departments) in 672 factories in 12 cities in China. A variety of industries and occupations using benzene were studied, including painting, printing, and manufacture of footwear, paint, and other chemicals. An unexposed comparison group was assembled from workers employed between 1972-87 in work units where benzene was not used in 69 of these factories and in 40 additional factories.(6,7) Subjects were identified from salary records and other factory written administrative records. We abstracted demographic data, including name, birth date, sex, and occupational data, including the dates of employment, by work unit and job title, for all jobs held by subjects in the study factories.

For benzene exposure assignment, we developed a standardized job-title dictionary comprised of 60 benzene exposure-specific job-titles in 11 major activity groups. Exposure assignments were made for benzene-exposed jobs by a factory exposure assessment team consisting of industrial hygienists, factory safety officers, and other employees, following a predetermined exposure assignment algorithm. Using available air measurements of benzene (n = 8,477) and abstracted data on benzene use and working conditions, benzene exposure was assigned in six concentration ranges (<1 ppm; 1-5 ppm; 6-10 ppm; 11-25 ppm; 26-50 ppm; and >50 ppm) for each job in seven calendar periods (1949-59, 1960-64, 1965-69, 1970-74, 1975-79, 1980-84, 1985+). Following exposure assignment, data abstraction forms were edited and reviewed to resolve discrepancies between benzene exposure estimates and abstracted exposure information.

Subjects were followed up for history of benzene toxicity, selected lymphohematopoietic malignancies and other hematologic disorders and for vital status to December 31, 1987 through factory personnel records at study factories and subsequent places of employment, or, as needed, by contacting next-of-kin, work colleagues, treating physicians, or others. For deceased subjects, cause of death was obtained from employer medical records, other written factory records or death certificates. Only after extensive search had failed to locate written records listing cause of death, were treating physicians or next-of-kin contacted.

For cases newly diagnosed with lymphohematopoietic malignancies and other hematologic disorders during the period of follow-up, pertinent histopathologic material, pathology reports and medical records were requested. For pathology review, clinical, laboratory, and pathologic data were abstracted onto standardized forms by physician investigators.

All histopathologic and bone marrow aspirate slides and peripheral blood smears were reviewed systematically, by expert hematopathologists affiliated with the Mayo Clinic and Peking Union Hospital, using structured abstract forms to objectively characterize hematopoiesis. Diagnoses were assigned after evaluation of all available clinical, laboratory, and pathologic data, without knowledge of the patient's benzene exposure status. Published criteria were used to categorize leukemia and lymphoma cases, and where possible to classify these cases and those with myelodysplasia (MDS).(8,9) For selected analyses, AML and MDS were combined as one disease category because of possible commonalities in the natural history of these conditions, and failure in the past to consistently classify MDS as entities distinct from AML.

For the statistical analysis, person-years were accumulated for the benzene-exposed workers from January 1, 1972 or, if hired later, from the first date of employment in a benzene-exposed job. For the unexposed comparison group, person-years were accumulated from January 1, 1972 or, if hired later, from the first date of employment. Analyses for mortality due to all causes and for incidence of lymphohematopoietic malignancies and other hematologic disorders were made by internal comparison of disease rates in the benzene-exposed group to the rates in the unexposed group, by Poisson regression analysis, yielding rate ratios (RR) for exposed versus unexposed workers, with control for age and, where appropriate, for sex.

Dose-response was assessed with respect to duration (<5, 5-9, 10 + years), average (<10, 10-24, 25 + ppm), and cumulative exposure (<40, 40-100, 100 + ppm-yrs) to benzene. For dose-response analyses, each measure of benzene exposure for each individual was allowed to change with time; person-years and disease events were assigned to benzene exposure levels with a 1.5 year lag (i.e., according to the level 1.5 year previously). In order to further evaluate the temporal component of disease development, we partitioned cumulative exposure at a given time into recent (1.5 years to 10 years earlier) and distant (10 or more years earlier) exposure. Because a substantial number of workers were exposed to a relatively stable average amount of benzene, we also developed a measure of constant exposure, where follow-up was censored 1.5 years after the individual's exposure level changed (< 10 ppm, 10-24 ppm, 25 + ppm) for the first time.

The study group consisted of 74,828 benzene-exposed and 35,805 unexposed workers. On average, benzene-exposed subjects were followed for 10.5 years, while unexposed subjects were followed for 11.7 years. Women contributed 47 percent of the person-years in the benzene-exposed study group and 40 percent in the unexposed group. Overall, the study groups were young, with about 60 percent of the total person-years at risk being contributed by subjects less than thirty years of age at study entry. About 2 percent of study subjects died during the follow-up period (1,369 benzene-exposed and 598 unexposed). Only 147 exposed and 90 unexposed workers were lost to follow-up.

There were 412 benzene poisoning cases among 62,234 exposed subjects (614,509 person-years) in 11 of the study cities. Risks for benzene poisoning increased with increasing intensity of benzene exposure at one and a half year prior to diagnosis of

benzene poisoning (< 5 ppm, RR = 1 [referent]; 5-19 ppm, RR = 2.2 (95%CI=1.7-2.9); 20-39 ppm, RR = 4.7 (95%CI=3.4-6.5); and 40 + ppm, RR = 7.2 (95%CI=5.3-9.8). Relative risks of benzene poisoning by cumulative exposure of 40-99 ppm-yrs, 100-399 ppm-yrs, and 400 and more ppm-yrs, respectively, were 1.7 (95%CI=1.3-2.3), 2.0 (95%CI=1.5-2.6), and 2.4 (95%CI=1.8-3.2), compared to subjects who had less than 40 ppm-yrs cumulative exposure to benzene.(10)

Eighty-one incident cases of lymphohematopoietic malignancies (n= 63) and other hematologic disorders (n= 18) were documented among benzene-exposed workers, with 13 lymphohematopoietic malignancies and no other hematologic disorders identified among the unexposed comparison group (1). Risk was significantly elevated for all lymphohematopoietic malignancies combined (RR= 2.6), malignant lymphoma (RR= 3.5) and leukemia (RR= 2.6). Among the leukemia subtypes, only AML was significantly elevated (RR= 3.1), although non-significant excesses were also noted for CML (RR= 2.6) and lymphocytic leukemia (RR= 2.8), the latter attributable to 5 cases of acute lymphocytic leukemia. Significant excess risks were also found for aplastic anemia and myelodysplastic syndrome. The relative risk for the rubric of acute myeloid leukemia plus MDS was RR= 4.1. The relative risk for all lymphohematopoietic malignancies and other hematologic disorders combined was 3.4 and for non-Hodgkin's lymphoma the RR= 3.0. No cases of Hodgkin's disease were identified. Disease risks were similar for men and women (11). Risk for ANLL/ MDS was substantially increased (RR= 70.6), however, among workers with a prior history of benzene poisoning (5).

Although occupation-specific results were limited by small numbers, risks for ANLL/MDS were elevated among coatings workers (RR= 4.2), rubber workers (RR= 6.1), chemical workers (RR= 4.5), and among those with other or mixed occupations (RR= 4.4) (3). Risk for NHL was also elevated among several occupational groups, but only chemical workers showed a statistically significant excess (RR= 7.8).

ANLL and ANLL/MDS both showed patterns of increasing risk with increasing average exposure to benzene, with more consistent exposure-response patterns for ANLL/MDS than for ANLL alone (3). The link of ANLL/MDS with average exposure was strongest when restricted to subjects with constant levels of exposure: risks rose from 3.2 for those with constant low level exposures (< 10 ppm) to 7.1 for those with constant high level exposures (25 + ppm). Risk for ANLL/MDS increased with increasing cumulative exposure to benzene, but the highest risks were not seen in the highest exposure level (cumulative ppm-years: 40-99, RR= 6.0; cumulative ppm-years: 100 +, RR= 4.4). Although there was some evidence of increased risk for leukemias other than ANLL among benzene-exposed workers, clear patterns of increasing risk with increasing exposure were not observed.

Risk of ANLL/MDS was six-fold and significantly increased among those who had only recent benzene exposure (14 exposed cases). Risk of ANLL/MDS was also strongly associated with increasing amounts of recent (p for trend= 0.003) but not distant exposure (p for trend= 0.51). NHL was strongly linked with distant exposure to benzene (p for

trend= 0.005, 13 exposed cases), but the association with exposure in the most recent 10 years was weak (p for trend= 0.15, 16 exposed cases).

Translational studies. Selected biomarkers were assessed among workers heavily exposed to benzene (n = 44; median benzene exposure: 31 ppm as an 8-hour TWA), among workers with a history of benzene poisoning (n= 50; benzene poisoning based upon routine hematologic exams), and among unexposed controls, in Shanghai, China.

Workers currently exposed to benzene had lower counts for white blood cells, lymphocytes, platelets, and red blood cells, and a lower hematocrit (4), however, lymphocyte count was the most sensitive indicator of benzene-induced hematotoxicity. Benzene air levels correlated with urinary levels of phenol, muconic acid, hydroquinone, and catechol, however, among those with the highest exposure to benzene, hydroquinone and muconic acid levels tended to plateau as a proportion of benzene metabolites.

Workers with a history of benzene poisoning were more likely than comparison controls to be rapid excretors of chlorzoxazone, an indicator of CYP2E1 activity, and to have two copies of the NQO1 609C-T mutation (combined OR= 7.6) (5). CYP2E1 and NQO1 enzymes are involved, respectively, in activation and detoxification of benzene and its metabolites, suggesting that genetic determinants of benzene metabolism may influence risk of benzene poisoning, which itself is a risk factor in our cohort study for ANLL/ MDS (RR= 70.6).

Acknowledgments. My collaborators in this work are: Drs. S-N Yin, G-L Li and L-Q Xi of the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, Beijing, China, Drs. Y-Z Wang and W Lu of the Station of Public Health and Prevention of Infection in Shanghai, China, Dr. Z-L Jiang of the Institute of Labor Health and Occupational Disease in Tianjin, China, Dr. T-R Dai of the Station of Public Health and Prevention of Infection in Chengdu, China, Dr. W-U Zhang of the Institute of Labor Health and Occupational Disease in Sichuan, China, Dr. X-J Chao of the Station of Public Health and Prevention of Infection in Chonqing, China, Dr. P-Z Ye of the Institute of Labor Health and Occupational Disease in Heilongjiang, China, Dr. Q-R Kou of the Institute of Labor Health and Occupational Disease in, Shenyan, China, Dr. Y-H Fan of the Institute of Labor Health and Occupational Disease in Jinzhou, China, Dr. X-C Zhang of the Institute of Labor Health and Occupational Disease in Henan, China, Dr. X-F Lin of the Institute of Labor Health and Occupational Disease in Guangzhou, China, Dr. J-F Meng of the Institute of Labor Health and Occupational Disease in Jiangxi, China, Dr. J-S Zho of the Institute of Labor Health and Occupational Disease in Nanchang, China, Dr. WJ Blot of the International Epidemiology Institute, Rockville, MD, Drs. MT Smith, L-P Zhang, S Campleman and N Titenko-Holland of the University of California, Berkeley, CA, Drs. Z-N Zhang and D-G Li of the Peking Medical College Hospital, Beijing, China, Dr. C-Y Li of the Mayo Clinic, Rochester, Minn, Drs D Ross and RD Traver of the University of Colorado, Denver, CO, , Dr. B Hoener of the University of California, San Francisco, CA, Dr. W Bechtold ot the Lovelace Inhalation Toxicology Research unit, Albuquerque, NM, and Drs. M Linet, N Rothman, S Wacholder, R N Hoover, W-H Chow

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PETROLEUM WORKER STUDIES AND BENZENE RISK ASSESSMENT

Robert Schnatter

Most epidemiologic studies involving benzene-exposed workers can be broadly classified as those that involve workers who used benzene-based solvents, and those that involve workers exposed to benzene from petroleum products. Risk assessments have focused upon the former (e.g., Rinsky et al., 1987), and have not directly used the results of the latter group of studies. However, these studies reflect exposure scenarios that are more like those of the general population. The focus of this presentation will be on the latter group of studies, and their potential use in comprehensive benzene risk assessments.

Workers in petroleum studies cover a broad time period stretching from the 1920's and earlier to the 1980's. The benzene content of gasoline has always varied (Garner and Evans, 1932), but probably rose somewhat following the introduction of catalytic cracking technology in the late 1930's. From that time forward, the benzene content of gasoline has typically been in the range of 1-5% with rarer instances of higher percentages noted. Worker benzene exposure from refinery operations probably increased from the 1920's to 1960's and then decreased over time due to fugitive emission control and other exposure control initiatives. Benzene exposures in distribution and retail operations also probably increased from the 1920's to 1950's, but then remained relatively stable thereafter. Typical eight hour exposure concentrations in distribution and retail operations average less than one part per million, although exposures typically reach 2-3 ppm for briefer (e.g., 30-120 minute) periods. These exposures are one to two orders of magnitude lower than those present in workers exposed to benzene through past solvent use (e.g., Rinksy et al., 1987).

Several similarly designed cohort studies have been reported in petroleum workers. Unfortunately, benzene exposure has not been directly measured for each study subject, although reasonable bounds can certainly be placed on benzene exposure in the aggregate study populations.

Three nested case control studies in petroleum workers have either been conducted or are nearing completion. These studies, also similarly designed, have conducted thorough benzene exposure assessments for each worker in the study populations. The first of these studies involved 29 Canadian lymphohematopoietic cancer cases (14 leukemias, seven multiple myelomas, and eight non-Hodgkin lymphomas) and 115 matched controls (Schnatter et al., 1996). Average daily benzene concentrations ranged from 0.01 to 6.2 ppm (Armstrong et al., 1996). Several exposure metrics and categories were analyzed for the three types of LH cancer. Limited information on confounders (e.g., smoking) was available. In general, there was no consistent dose response relationship found for any of the three types of LH cancer. Benzene exposure duration showed a slightly stronger association with leukemia than other metrics such as cumulative exposure, mean intensity, and the number of years above either 0.5 or 1 ppm benzene exposure. The results are consistent with either a lack of effect due to benzene, or inadequate power to detect an effect as high as two-fold.

A second, larger study in U.K. workers was modelled after the Canadian study (Rushton and Romaniuk, 1997). This study involved 90 cases (all were leukemia) and 354 matched controls. Cases were identified and classified through both death certificates and cancer registry information with respect to leukemia cell type. For lymphatic leukemias as well as chronic myelogenous leukemia (CML), there were no consistent dose response trends for various exposure metrics. Results for acute myeloid leukemia (AML) were more difficult to interpret. There was a

suggestive dose response trend between AML and for categorical, but not continuous, exposure metrics. The dose response trend became slightly stronger when incorporating a five or ten year lag between exposure and outcome, or when incorporating peak exposure metrics. However, in sensitivity analyses, the dose response trend between AML and categorical metrics became weaker, and indeed disappeared when only the strongest exposure estimating information was used (i.e., complete work history records, and exposures given higher confidence scores). The authors interpreted the study as weakly suggestive of an effect, but noted that because the study lacked internal consistency, no firm conclusions could be made.

A third study is underway in Australia. It is due to be completed in 2000 and will consist of approximately 80 cases of LH cancer and matched controls. Again, benzene exposure estimates will be quantified, and regression analyses will be performed. An advantage of the Australian study is that relatively good information on smoking will be available, and a greater fraction of cases and controls were employed in more recent time periods, perhaps allowing more certainty in resulting benzene exposure estimates.

In risk assessment jargon, these studies do not demonstrate that benzene is a "hazard" at typical workplace concentrations. Thus, although it is mathematically possible, it would not be appropriate to calculate unit risk estimates for benzene's potency. However, the studies can be used to provide some reassurance that typical benzene exposure concentrations experienced by petroleum workers are not causing large increases of AML or other leukemias. Certainly, they do not provide any justification for lowering occupational workplace limits below 1 ppm.

The petroleum cohort studies also have some role in benzene risk assessments. Their strengths and weaknesses are opposite those of the case control studies. The case control studies have measured exposure as precisely as possible; the cohort studies have not. Small numbers limit the case control studies, but the aggregate cohort population is very large. However, both sets of studies do have a role in comprehensive benzene risk assessments.

Wong and Raabe (1995) have summarized cell-specific leukemia mortality in U.S. and U.K. petroleum worker studies. The analysis covered 19 studies, more than 208,000 workers, and more than 4.5 million person years of observation from 1937 through 1989. There were 327 leukemia deaths that were classified by cell type. Most of the aggregate population consisted of refinery workers, with smaller groups of distribution, marine, and upstream workers also included. While no formal analysis of heterogeneity was performed, the authors thought it was likely that within-cohort exposure variation would be comparable, if not greater than, between-cohort exposure variation. Overall SMR's and 95% confidence intervals for the four major leukemia cell types were as follows: AML - 0.96 (0.81 - 1.14), CML - 0.82 (0.62 - 1.08), ALL - 1.22 (0.83 - 1.71), and CLL - 0.79 (0.62 - 1.00).

While these results also do not suggest a hazard from benzene at these exposure concentrations, benzene exposures were not directly measured in any of the petroleum worker cohort studies. However, reasonable estimates of exposure can be made using comprehensive industry-wide surveys of benzene exposure. Runion and Scott (1985) reported over 14,000 benzene samples submitted by over 30 companies for refinery operations. For a relatively recent time period (1978-83), exposures averaged 0.22 ppm. Using another extensive data set from Tsai (1983), which covers 737 samples from a somewhat earlier period (1973-82), a higher mean of 0.65 ppm can be estimated. Some of the difference in these two numbers is probably attributed to advances in fugitive emission control in the 1970's. The average from both data sets yields an

estimate of 0.44 ppm, which may still be somewhat of an underestimate for a population that extends back into the 1930's. However, there are undoubtedly some workers in the study population who were exposed to only background benzene levels. Thus, an estimate of 0.44 ppm for the average exposure in the population appears reasonable.

Hertz-Piccioto (1995) summarized how epidemiologic data can be applied in quantitative risk assessment. For a study or studies to be used "as a basis for extrapolation", or as a basis for potency calculations, she suggests that four criteria be met: (a) a moderate to strong positive association be present, (b) strong biases are unlikely, (c) confounding is limited, and (d) quantification of exposure be linked to individuals. For the case control studies (a) is lacking, while for the cohort studies, both (a) and (d) are lacking. For a study to be used to "check plausibility", she suggests a moderate to strong positive association is often not required. The studies, however, should at least partially be able to rule out confounding and bias. In addition, some quantification of exposure is needed "even if based on data external to the study site". Clearly, both the case control studies and the cohort studies in petroleum workers would qualify for use as "plausibility checks".

Because existing unit risk estimates for benzene exposure are based on a cohort study, the petroleum cohort studies are more amenable for use as plausibility checks. The Wong and Raabe (1995) aggregate population was exposed to benzene in an occupational scenario. Unit risk estimates have also been developed in workers who were exposed to benzene in the occupational environment. Frequently, a first step in converting study results from these scenarios to the general population is to apply dosimetric adjustments, taking into account the number of days per year, hours per day, and number of years exposed. If unit risk estimates (of 6 per million) are applied to the Wong and Raabe population, and dosimetric adjustments are made, 163 excess deaths due to AML would be predicted. These deaths along with the 155 expected deaths would predict 318 total deaths due to AML in the Wong and Raabe population. However, the Wong and Raabe confidence limits can be used to calculate that the study is consistent with from 125 to 175 total AML deaths. The 318 deaths falls well outside of this range, and therefore, is inconsistent with the results of Wong and Raabe (1995).

There are a few ways to resolve this inconsistency. The first would be to reject the previous unit risk estimates, recognize the lack of effect in petroleum workers, and apply appropriate uncertainty factors applicable to environmental, rather than occupational, exposure scenarios. There is some scientific justification for this approach, as benzene exposure in the petroleum workers is more analogous to benzene exposure in the general population, the level of benzene exposure in petroleum workers is closer to general population exposures, necessitating less extrapolation. A second approach, which is more of a compromise, would be to calculate a unit risk estimate consistent with the upper confidence limit of the Wong and Raabe study. A third approach would be to ignore the inconsistency, and continue to use the unit risk factor for solvent-exposed populations. This may be the least scientifically supportable approach.

In summary, the existing studies in petroleum workers qualify for use as plausibility checks on benzene unit risk estimates. When the studies are used in this manner, they indicate that existing unit risk estimates of benzene's potency are not consistent with this body of literature. Therefore, procedures which integrate the petroleum worker studies and benzene solvent-user studies are indicated.

Biomarkers in the Molecular Epidemiology of Benzene-Exposed Workers

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Introduction

Biomarkers can be classified into three categories: biomarkers of exposure, susceptibility, and early effect. Along with colleagues from the National Cancer Institute, the Chinese Academy of Preventive Medicine in Beijing, the Shanghai Anti-epidemic Center, the University of North Carolina and other institutions in the United States, we have applied various biomarker methods to samples obtained from workers exposed to high levels of benzene. The goal of these studies is to develop and validate 1) biomarkers of exposure to benzene, such as albumin or hemoglobin adducts; 2) molecular markers of susceptibility to benzene, such as inherited polymorphisms in enzymes involved in the metabolism of benzene; and 3) biomarkers of the early effects of benzene, including hematotoxicity (complete blood cell counts), gene mutations (glycophorin A), and chromosome aberrations detected by fluorescence in situ hybridization (FISH), G-banding and the micronucleus assay. An introduction to these studies has been presented previously • ADDIN ENRfu ••(Rothman, et al., 1996a; Rothman, et al., 1996b; Smith and Rothman, 1998; Smith and Zhang, 1998)•, and only those findings pertaining to biomarkers of exposure and early effects will be discussed here.

Sample Collection and Exposure Assessment

Biological samples were collected from 44 healthy workers currently exposed to benzene with minimal exposure to toluene and other aromatic solvents in Shanghai, China in October, 1992. The same number of healthy controls without current or previous occupational exposure to benzene were enrolled from factories in the same geographic area. Controls were frequency-matched by gender and age (5 year intervals). Exclusion criteria for all subjects were history of cancer, therapeutic radiation, chemotherapy, or current pregnancy. Each subject was administered a questionnaire by a trained interviewer. Data collected included age, gender, current and lifelong tobacco use, current alcohol consumption, medical history and work history. Height and weight of each subject were measured and peripheral blood was obtained by phlebotomy.

Individual exposure was monitored by organic vapor passive dosimetry badges (3M #3500, St. Paul, Minnesota), which were worn by each worker for a full workshift on 5 separate days during the one- to two-week period prior to phlebotomy. An eight-hour time weighted average (TWA) exposure was calculated for benzene as the geometric mean of the five air measurements. Cumulative exposure to benzene was calculated by multiplying historical time-specific exposure estimates by the duration worked. All exposure assessment was performed blinded with respect to the biomarker analysis.

Biomarkers of Exposure

The median benzene air level among the exposed workers was 31 ppm as an 8 hour TWA (range: 1-328 ppm). Air monitoring data were confirmed by measures of urinary benzene metabolites; phenol, muconic acid, catechol and hydroquinone showed strong, positive correlations with air benzene levels, and were substantially higher in exposed workers compared to controls • ADDIN ENRfu ••(Rothman, et al., 1998)•. In addition, current benzene air levels were inversely correlated with the absolute lymphocyte count among exposed workers, consistent with

previous reports that lymphocytes are particularly sensitive to benzene • ADDIN ENRfu ••(Rothman, et al., 1996a; 1996b)•. The initial metabolite of benzene, benzene oxide (BO), reacts with cysteinyl residues in hemoglobin (Hb) and albumin (Alb) to form protein adducts (BO-Hb and BO-Alb), which are presumed to be specific biomarkers of exposure to benzene. With Dr. Stephen Rappaport's lab in Chapel Hill we analyzed BO-Hb in 43 of the exposed workers and 42 unexposed controls and BO-Alb in a subsample consisting of 19 workers and 19 controls • ADDIN ENRfu ••(Yeowell-O'Connell, et al., 1998)•. The adducts were analyzed by GC-MS following reaction of the protein with trifluoroacetic anhydride and methanesulfonic acid. When subjects were divided into controls (n = 42) and workers exposed to < 31 ppm (n = 21) and > 31 ppm (n = 22) benzene, median BO-Hb levels were 32.0, 46.7 and 129 pmol/g globin, respectively (correlation with exposure: Spearman $r = 0.67$, $p < 0.0001$). These results represent the first observation in humans that BO-Hb levels are significantly correlated with benzene exposure. Median BO-Alb levels in these 3 groups were 103 (n = 19), 351 (n = 7) and 2010 (n = 12) pmol/g Alb, respectively, also reflecting a significant correlation with exposure (Spearman $r = 0.90$, $p < 0.0001$). These results clearly affirm the use of both Hb and Alb adducts of BO as biomarkers of exposure to high levels of benzene.

Biomarkers of Early Effect from Benzene Exposure

A potential method of predicting who is most at risk for benzene-induced leukemia is to determine the extent of the genetic damage it produces in exposed individuals, using biomarkers of early effect. One means of assessing genetic damage is to measure mutations in specific genes, such as glycophorin A (GPA) • ADDIN ENRfu ••(Compton, et al., 1991; Jensen and Bigbee, 1996)•. An increased level of "gene-duplicating" mutations in GPA was found in the benzene-exposed workers • ADDIN ENRfu ••(Rothman, et al., 1995)•. Interestingly, this increased mutation frequency was correlated with cumulative exposure to benzene. Since cumulative exposure to benzene may correlate best with leukemia risk, the GPA assay appears to have potential as a biomarker of early biological effect for benzene and other leukemogens. The GPA assay has drawbacks, however. First, it is relatively insensitive: high benzene exposure (mean time-weighted average (TWA) at 72 ppm) only elevated the combined mutant frequency from 16.3 to 23.0 per million, a 41% increase • ADDIN ENRfu ••(Rothman, et al., 1995)•. Second, it can only be performed on GPA heterozygous (type MN) individuals, who statistically constitute only 50% of any given population under study. Thus, although the GPA assay can provide important mechanistic information, it may not be an ideal biomarker of early effect. The most common means of detecting genetic damage has traditionally been conventional cytogenetics. Numerous publications have demonstrated a clear association between benzene exposure and increased levels of chromosome aberrations in peripheral blood cells. Since chromosome aberrations in peripheral blood lymphocytes have been shown to be associated with increased risk for overall cancer incidence • ADDIN ENRfu ••(Hagmar, et al., 1994)•, especially for increased mortality from hematological malignancies • ADDIN ENRfu ••(Bonassi, et al., 1995)•, it is possible that specific chromosome aberrations may provide even better markers of future leukemia risk.Specific Chromosome Aberrations in Leukemia

Specific chromosome aberrations are the hallmark of human leukemia • ADDIN ENRfu ••(Hagemeijer and Grosveld, 1996)•. Aneuploidy, the loss or gain of specific chromosomes in AML and MDS (such as trisomy 8 and monosomy 5 and 7), is commonly observed as specific chromosome translocations, inversions and deletions (e.g. t(8;21), t(9;22), inv(16) and del(5q)) • ADDIN ENRfu ••(Hagemeijer and Grosveld, 1996)•. The loss of chromosomes 5 and 7 and their long-arm deletions are the two most common changes in therapy-related AML and MDS (t-AML and t-MDS), especially among patients previously treated with alkylating agents • ADDIN ENRfu ••(Pedersen-Bjergaard, et al., 1995)•. Treatment with topoisomerase II inhibitors is

associated with balanced chromosome aberrations such as t(8;21) and t(11q23) in t-AML • ADDIN ENRfu ••(Pedersen-Bjergaard, et al., 1995)•. These specific chromosome aberrations are also more common among leukemia patients with previous exposure to chemical solvents (including chronic exposure to benzene, insecticides, petroleum, etc.) • ADDIN ENRfu ••(Crane, et al., 1996; Mitelman, et al., 1981)•. Detection of Specific Chromosome Aberrations by FISHWe have applied FISH to determine the presence of specific chromosome aberrations in the lymphocytes of otherwise healthy workers exposed to benzene and matched controls. Initially, we studied hyperdiploidy levels of chromosome 9 in interphase cells because trisomy 9 has been observed in benzene-poisoned patients • ADDIN ENRfu ••(Erdogan and Aksoy, 1973; Forni and Moreo, 1967)• and benzene metabolites induce hyperdiploidy of this chromosome in cultured lymphocytes in vitro • ADDIN ENRfu ••(Eastmond, et al., 1994; Zhang, et al., 1994)•. High benzene exposure was shown to increase hyperdiploidy of chromosome 9 in the lymphocytes of otherwise healthy workers, with trisomy 9 being the most prevalent form • ADDIN ENRfu ••(Zhang, et al., 1996)•. We have gone on to use interphase cytogenetics to study the hyperdiploidy of chromosomes 7 and 8 and these findings will be published shortly. Interphase cytogenetics cannot be used, however, to detect monosomy or rare translocations because of artifacts related to probe overlap • ADDIN ENRfu ••(Eastmond and Pinkel, 1990)•. Monosomy of chromosomes 5 and 7 and translocation (8;21) are among the most common aberrations observed in AML. We therefore used chromosome-painting and region-specific fluorescent probes to examine AML-specific aberrations, including -5, -7, del (5q31), del (7q22-34) and t(8;21), in metaphase spreads prepared from the lymphocytes of workers exposed to benzene and matched controls • ADDIN ENRfu ••(Smith, et al., 1998; Zhang, et al., 1998a)•. We painted chromosomes 8 and 21 in lymphocyte metaphases from the 44 workers exposed to benzene and 44 matched controls. To examine dose-response relationships the workers were divided into 2 groups at the median exposure level, a lower-exposed group (= 31 ppm, n = 21) and a higher-exposed group (> 31 ppm, n = 22). Benzene exposure was associated with significant increases in hyperdiploidy of chromosome 8 (1.2, 1.5, 2.4 per 100 metaphases; Ptrend < 0.0001) and 21 (0.9, 1.1, 1.9; Ptrend < 0.0001). Translocations between chromosomes 8 and 21 were increased up to 15-fold in highly exposed workers (0.01, 0.04, 0.16; Ptrend < 0.0001). In one highly exposed individual these translocations were reciprocal and were detectable by reverse-transcriptase PCR • ADDIN ENRfu ••(Smith, et al., 1998)•. These data indicate a potential role for t(8;21) in benzene-induced leukemogenesis and are consistent with the hypothesis that detection of specific chromosome aberrations may be a powerful approach to identify populations at increased risk of leukemia from benzene exposure.

We also used a novel FISH procedure to determine if specific aberrations in chromosomes 1, 5 and 7 occured at an elevated rate in metaphase spreads prepared from the lymphocytes of the same benzene-exposed Chinese workers • ADDIN ENRfu ••(Zhang, et al., 1998a)•. We found that benzene exposure was associated with increases in the rates of monosomy 5 and 7 but not monosomy 1 ($p < 0.001$; < 0.0001; and 0.94, respectively) and with increases in trisomy and tetrasomy frequencies of all three chromosomes. Long arm deletion of chromosomes 5 and 7 was increased in a dose-dependent fashion ($p = 0.014$ and < 0.0001) up to 3.5-fold in the exposed workers. These results demonstrate that leukemia-specific changes in chromosomes 5 and 7 can be detected by FISH in the peripheral blood of otherwise healthy benzene-exposed workers. We have also shown that benzene metabolites induce these same changes in vitro • ADDIN ENRfu ••(Zhang, et al., 1998b)•. Taken together all of these data obtained using FISH suggest that aberrations in chromosomes 5, 7, 8 and 21 may be a useful biomarker of early biological effect for benzene exposure. Indeed, Dr. Luoping Zhang of Dr. Smith's laboratory has recently devised a FISH procedure to examine the leukemia related changes in all 4 of these chromosomes

simultaneously. Studies are being planned to apply these methods in workers exposed to a broad range of benzene concentrations. In addition, we will explore the impact of interindividual variation in genes that activate and detoxify benzene and its metabolites on these outcomes, following up our previous report that variation in CYP2E1 and NQO1 influence the risk of benzene hematotoxicity • ADDIN ENRfu ••(Rothman, et al., 1997)•. AcknowledgementsWe are indebted to the study subjects and the staff of the Chinese Academy of Preventive Medicine, Beijing, and the Shanghai Hygiene and Anti-Epidemic Center, Shanghai, China for their assistance and participation in this study. These studies were supported by funds from the National Cancer Institute and National Institute of Environmental Health Sciences (Grants RO1 ES06721, P42 ES04705 and P30 ES01896) and the California Environmental Protection Agency. The following individuals have contributed greatly to these studies: Luoping Zhang, Nina Titenko-Holland, Yunxia Wang, Prema Kolachana, Joseph Wiemels, Kathleen Meyer (School of Public Health, University of California, Berkeley, CA 94720); Richard Hayes, Mustafa Dosemeci (Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD 20892); Songnian Yin, Guilan Li, Liqiang Xi, Weihong Guo (Chinese Academy of Preventive Medicine, Institute of Occupational Medicine, Beijing, China); Stephen Rappaport, Karen Yeowell O'Connell, Suramya Waidyanatha (School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill NC 27599-7400); Yao-Zhu Wang, Lu Wei (Shanghai Hygiene and Anti-Epidemic Center, Shanghai, China); and Robert Haas (California EPA).

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Abstract for " Benzene: State-of the Science Workshop", Ottawa, Canada, December 16-17, 1998

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THE HEALTH EFFECTS INSTITUTE'S BIOMARKER RESEARCH IN CHINA. Martha E. Richmond, Health Effects institute, Cambridge, MA.

Exposure to benzene is associated with the development of several blood disorders, including aplastic anemia, pancytopenia, chromosomal aberrations, and acute non-lymphocytic leukemia (Aksoy, 1989; Crump, 1994; Forni, 1996; Hayes et al., 1997; Rinsky et al., 1987; Rothman et al., 1996). Much of the evidence establishing the relationship between benzene exposure and the development of these disorders has come from occupational health studies where exposures generally range from 2 to 100 ppm. However, the magnitude of risk for adverse health effects at ambient levels of exposure, which range from <1 to 3 ppb, remains to be addressed. In particular, information about the mechanism of leukemogenesis and the development of aplastic anemia in human populations is incomplete. There is clearly a threshold for benzene-induced aplastic anemia, a condition characterized by a decrease in the number of circulating red and white blood cells and platelets accompanied by almost complete replacement of many bone marrow cell types by scar tissue. However, it is still unknown whether blood cell toxicity, manifested as aplastic anemia, is a necessary step in the development of leukemia. Also limiting an understanding of leukemia is a significant knowledge gap about the cellular mechanisms for benzene-induced leukemia, especially the steps required in the early stages.

The existence of knowledge gaps is an important public health issue, since there are a number of environmental sources for benzene exposure, ranging from main and side-stream tobacco smoke to certain food stuffs (Wallace, 1996). Benzene is present in gasoline, ranging from somewhat less than 1% in the United States (AAMA, 1995) up to approximately 5% in some other countries. Benzene is named as one of the mobile source air toxics in the Clean Air Act Amendments of 1990. In response to provisions of the Clean Air Act Amendments the Health Effects Institute (HEI) held a workshop in 1992 which formed the basis of a publication issued in 1993, "Research Priorities for Mobile Air Toxics". This document identified research needs related to benzene and several other mobile source air toxics. Specific research needs for benzene included the need to: (1) develop sensitive methods to measure metabolites from low-level exposure to benzene; (2) identify markers of exposure, uptake, and metabolism and validate these markers in human populations; (3) develop dose-response information on biomarkers so as to evaluate the relationship of exposure levels to levels of metabolites and genetic changes within and across species; (4) make progress toward elucidating the mechanism of benzene toxicity.

As a first step in addressing several of these research needs, HEI funded a study looking at methods to measure metabolites from low-level exposure to benzene. HEI also funded several biomarker development studies. Two of these studies were to develop biomarkers of exposure

and dose, and the third to develop a biomarker of effect:

C S-phenylcysteine in albumin as a benzene biomarker, a marker of exposure developed by Dr. William Bechtold of the Lovelace Respiratory Research Institute

C Development of liquid chromatographic-electrospray ionization-mass spectrometric methods for determination of urinary metabolites of benzene, markers of exposure and dose developed by Dr. Asseih Melikian of the American Health Foundation. Specific metabolites include t,t-muconic acid, S-phenylmercapturic acid, 1,2,4-trihydroxybenzene, hydroquinone, and catechol.

C Characterization and mechanisms of chromosomal alterations induced by benzene in mice and humans. This study includes development of methods to measure chromosomal alterations, biomarkers of effect, using fluorescent in situ hybridization (FISH) developed by Dr. David Eastmond of the University of California at Riverside.

The original developmental work of the biomarkers was done in animal systems. However, the goal of the studies was to develop markers that would be useful in humans exposed to ambient levels of benzene. To achieve this second goal, HEI issued a request for qualifications (RFQ) in 1995 to establish a collaborative relationship between investigators with access to benzene-exposed populations and investigators who had developed the biomarkers in animal systems. The relationship would result in a proposal for a transitional epidemiology study to validate the biomarkers in human populations. As the result of this process, HEI is now funding a study under the overall direction of Dr. Qingshan Qu of the Institute of Environmental Medicine at New York University. This study is being conducted in collaboration with investigators from the Chinese Academy of Preventive Medicine under the direction of Dr. Guilan Li.

Dr. Qu's study involves sub-sets of a cohort of occupationally exposed benzene workers and unexposed workers identified by the National Cancer Institute (Yin, et al., 1994). The study is divided into two phases. The focus of the first phase is to determine sensitivity, reproducibility, and the time course of elimination of biomarkers of exposure and dose. The focus of the second phase is to develop dose-response profiles of all markers determined to be appropriate based on results of the first phase. Phase I is now nearing completion. Phase II is under way and expected to end late in 1999.

A total of 25 exposed subjects and 25 control subjects were enrolled in Phase I. Benzene-exposed workers were employed in glue and shoe manufacturing facilities in Tienjing Province, China. In multiple exposure assessments, benzene exposures ranged from 10 ppm to 150 ppm with a median ranging from 19-26 ppm depending on the occasion of

exposure measurement. There were also small amounts of xylene and toluene present in the glue and shoe manufacturing facilities (approximately 5-10% of the benzene concentration). Unexposed controls were workers employed in a food processing facility in the same region of China. Exposure of controls was below the level of detection in personal and area sampling methods. Current exposure of workers was determined by personal samplers, and historical exposure profiles for all subjects has been determined from work records. In Phase I, all enrolled subjects wore duplicate personal samplers.

Blood and urine samples, taken from all enrolled subjects were distributed to laboratories of Drs. Bechtold, Eastmond and Melikian for analysis of biomarkers. To gain more information about the metabolite or mixture of metabolites associated with short-term exposure, urine samples collected before and after work shifts were analyzed for several markers of exposure. A time-course study of the urinary biomarkers was also conducted to determine the kinetics of elimination. Markers included the benzene-specific marker, S-phenylmercapturic acid, as well as t,t-muconic acid, benzene triol, hydroquinone and phenol. While some of these markers are not specific to benzene, several are thought to be related to benzene toxicity (Gut, et al., 1996; Smith, 1996; Witz et al., 1996), and information about their levels and patterns, especially at the lower levels of exposure, may provide important information about benzene metabolism. Blood samples were analyzed for S-phenylcysteine adducts of albumin as a biomarker of longer term exposure. Blood samples were also analyzed for chromosomal breakage and hyperdiploidy of interphase lymphocytes and Go granulocytes using FISH. Metaphase chromosomes from lymphocytes have also been analyzed for chromosomal aberrations of all chromosomes using more conventional techniques. Routine clinical analyses of all blood samples were conducted for hemoglobin, red blood cells, red blood cell morphology and white blood cells. A differential analysis of all white blood cells was also conducted. Data analysis will correlate exposure levels with the various markers of exposure and effect. Data analysis will also correlate a number of end points as a means of beginning to establish links between potential toxic effects (as determined by chromosomal aberrations and blood count data) and markers of exposure. Final data analysis of Phase I results is nearing completion.

At the present time, the dose-response study (Phase II) is underway. A total of 130 subjects are enrolled in this phase. This includes 25 control subjects and 105 benzene-exposed subjects employed in manufacture of sporting goods. All subjects are from the Tienjing Province area of China. Benzene exposures range from < 1ppm to >15 ppm. Subjects are grouped into four exposure levels as follows: <1 ppm, 40 subjects; 1.1 to 5 ppm, 30 subjects; 5.1 to 15 ppm, 25 subjects, and >15 ppm 10 subjects. Based on results from Phase I, aspects of the end points and study design of Phase II have been modified. This includes the personal sampling strategy. Because of the excellent agreement

found in the analysis of duplicate personal samplers taken from Phase I subjects, a smaller number of the subjects enrolled in Phase II (10%) wore duplicate samplers. Personal exposure monitoring was conducted twice: once prior to obtaining biological samples; and once during the biological sampling. Monitors were analyzed both in China and at New York University. Because analysis of several selected chromosomes using FISH technology did not discriminate between exposed and unexposed workers, more conventional techniques, also used in Phase I, are being used to analyze lymphocytes for chromosomal aberrations, and all chromosomes will be examined. Phase II will also involve reconstruction of historical exposure profiles for all participating subjects, using work records.

Results of the biomarker validation study are expected to address several of the goals originally identified in HEI's benzene research program. Information should indicate whether the markers of short-term (urinary metabolites) and longer-term (albumin adducts) exposure are sufficiently sensitive to be used as markers of exposure to ambient environmental levels of benzene. Dose-response information for both biomarkers of exposure and biomarkers of effect will be developed and analyzed for possible relationships. This may provide additional information about relationships between exposure, metabolism, and genetic change. Finally, it may be possible, in a population-based study to identify certain individuals who may be more susceptible to toxic effects of benzene, as determined by chromosomal aberrations and blood count measurements.

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Recent Findings and New Initiatives for Epidemiologic Research on Benzene

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Presented at
1998 Benzene State of the Science Workshop
University of Ottawa
Ottawa, Canada
December 17, 1998

Summary of presentation

Recent epidemiologic research on benzene consists of (1) updated analyses of workers exposed to benzene at two facilities in Ohio, who were involved in the manufacture of Pliofilm®, (2) analyses of workers in the petroleum industry in North America, Australia and Europe, who were exposed to benzene and other petroleum products, and (3) workers from a broad spectrum of industries in China, who were exposed to benzene and a wide variety of chemicals.

Results from the original Pliofilm® study were reported by Infante et al. (1978), and Rinsky et al. (1981 and 1987). Based on mortality ascertainment through 1981, Rinsky et al. (1987) reported a significant increase in risk as well as an exposure-response relationship between cumulative benzene exposure and leukemia (all cell types combined). An increased risk of multiple myeloma was also reported, but no exposure-response relationship was found.

Subsequently, mortality in the Pliofilm® cohort was updated through 1987. Based on an analysis on leukemia (all cell types combined), Paxton et al. (1994) concluded that a significant increase in leukemia risk occurred at exposure above 50 ppm-years. However, based on an analysis of acute myeloid leukemia (AML), Wong (1995) concluded that a significant increase in AML occurred at a much higher level (200 ppm-years based on the set of lowest estimates), and that the slope of the exposure-response curve was much steeper. No additional death from multiple myeloma was reported in the update, and the risk was no longer significant. To examine the effect of an alternative exposure metric, Schnatter et al. (1996a) analyzed the Pliofilm® study using the long-term average concentration associated with the maximally exposed job of each worker. Using the lowest exposure estimates, Schnatter et al. (1996a) concluded that the critical concentration of benzene exposure for an increased risk of AML was between 20 and 25 ppm.

Benzene was the principal exposure at the Pliofilm® plants, and exposure levels were relatively high. Exposure estimates in the Pliofilm® study have been subjected to extensive

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review, and three different sets of estimates have been developed. Because of the relatively high exposures and the large number of AML cases, the Pliofilm® study will likely continue to provide valuable information on benzene epidemiology, especially for AML. The cohort is now ready for a 10-year update. However, discrepancies in exposure estimates have and will continue to hamper the interpretation. In addition, the small number of cases of non-AML leukemias limits the statistical power of the study.

Cohort studies of petroleum workers (primarily refinery and distribution workers) have been updated periodically. These workers were exposed to benzene and benzene-containing products. Unfortunately, few studies of petroleum workers have quantitative benzene exposure estimates, and job categories and duration of employment have often been used as surrogates for exposure. Exceptions were studies based on distribution workers in the US, Canada and the UK. In these studies, estimates of exposure to gasoline (in terms of total hydrocarbons) or benzene were provided (Smith et al., 1993; Wong et al., 1993; Schnatter et al., 1996b; Rushton and Romaniuk, 1997; Wong and Raabe, 1997a; Wong et al, 1999). In general, benzene exposure levels in the petroleum industry are relatively low, especially after 1950. For example, based on more than 1,700 samples collected between 1973 and 1982 at a large refinery in Texas, 89% full samples (sampling time > 4 hours, with the majority > 7 hours) were less than 1 ppm, 10% between 1 and 10 ppm, and only a few samples above 10 ppm (Tsai et al., 1983).

Overall reviews and meta-analyses by cancer site of petroleum studies have been reported previously (Wong and Raabe, 1989, 1995 and 1997b). For cell-type specific leukemias and other lymphopoietic cancers such as multiple myeloma, because of the small number of deaths in each study and the broad and overlapping categories used in US mortality statistics, separate results were not reported in many original reports of cohort studies in the US. Recently Wong and Raabe (1995 and 1997) reported results for cell-type specific leukemias and multiple myeloma for individual studies as well as for the combined database, which consisted of more than 250,000 workers and covered an observation period of 55 years (1937-1991). No increased mortality from cell-type specific leukemias or multiple myeloma was found.

These cohort studies of petroleum workers (particularly those in the US, Canada and Australia) will continue to be updated in the future. For large cohorts, nested case-control studies should be considered. Whenever possible, a thorough exposure assessment should be an integral part of all nested case-control studies. Currently, several cohort studies are being updated. In addition, an overall review and an updated meta-analysis by cancer site of all cohort studies in the petroleum industry are now underway.

In 1979-1981 the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine (CAPM), carried out a national survey in China, in which more than 500,000 workers were identified to have been exposed to benzene, and a benzene poisoning prevalence of 0.51% was reported (Yin et al., 1987a). The 95% range of benzene concentrations at workplaces were 0.06-844.74 mg/m^3 (0.02-266 ppm). Subsequently, 28,460 of these exposed workers from a variety of industries in 12 cities and 28,257 unexposed workers from the same cities were included in a historical cohort study (the "CAPM" study). The mortality of the cohort was observed between 1972 and 1981, and the results were reported in a number of publications (Yin and Li, 1986; Yin et al., 1987b; Yin et al., 1989). A total of 25 leukemia deaths and 5 incidence cases were reported among the exposed workers, while 4 deaths among the unexposed workers were attributed to leukemia. The standardized mortality ratio (SMR) of leukemia for the exposed workers in comparison to the unexposed workers was 5.74 ($p < 0.01$). No exposure-response

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analysis was performed, but the authors reported actual benzene measurements from locations where leukemia deaths and cases were observed (Yin and Li, 1986; Yin et al., 1987b; Yin et al., 1989). Exposure levels reported were as high as 5,508 mg/m^3 or 1,732 ppm.

Since 1987, the US National Cancer Institute (NCI) has collaborated with CAPM in expanding and updating the Chinese benzene study. The previous study was expanded to include 74,828 benzene-exposed workers and 35,805 unexposed workers who were employed for any length of time between 1972 and 1987 at 712 factories in 12 cities (the "CAPM-NCI" study). The general methods and resources of the expanded study were described by Yin et al. (1994). In a second paper, an historical benzene exposure assessment to characterize benzene exposure profiles of the cohort members was outlined (Dosemeci et al., 1994).

Based on comparisons between the exposed and unexposed workers, increased mortality risk ratios (RR) were reported for a variety of cancers: 2.4 for nasopharyngeal cancer, 1.8 for esophageal cancer, 1.2 for cancer of the liver and gall bladder, 1.4 for lung cancer (1.5 in men and 1.0 in women), 1.3 for brain tumors, 4.5 for lymphoma, and 2.3 for leukemia (Yin et al., 1996). Some exposure-response analyses in terms of cumulative exposure (ppm-years) for selected disease categories were reported by Hayes et al. (1996). Both lung cancer and the broad category of hematopoietic malignancies showed a significant upward trend ($p = 0.01$). For workers with more than 400 ppm-years of benzene exposure, the RR for lung cancer was 1.7 and that for hematopoietic malignancies was 2.0.

In another paper, Hayes et al. (1997) reported dose-related incidence of hematologic neoplasms. For leukemia, the RR was 2.5 (95% CI: 1.2-5.1). For acute non-lymphocytic leukemia (ANLL), the RR was 3.0 (95% CI: 1.0-8.9). The RR for non-Hodgkin's lymphoma (NHL) was 3.0 (95% CI: 0.9-10.5). The authors reported significant upward trends for ANLL with increasing average exposure, and with increasing constant exposure. The trend between ANLL and cumulative exposure was close to, but did not achieve, statistical significance ($p = 0.06$). For NHL, significant trends were reported for average exposure, duration of exposure and cumulative exposure. The highest NHL risk (RR = 7.8) was observed among "chemical" workers, which included "organic, insecticide, and benzene production workers."

Although the Chinese benzene study was one of the largest studies in terms of number of exposed workers, some results were based on comparisons to small numbers of cases for disease categories of interest in the unexposed group. For example, there were only 4 ANLL cases and 3 NHL cases among the unexposed workers. For the Chinese benzene study, results depended on not only the number of deaths in the exposed group but also the number of deaths in the unexposed group.

The exposed workers were employed in a variety of industries. In the CAPM study, 64% of the exposed workers were either painters or paint production workers (Yin et al., 1987b). In terms of exposures, only 5% were exposed to benzene only (Yin et al., 1987a). The potential problem of concomitant exposures complicated the interpretation of the results in relation to benzene exposure (for example, the NHL increase in workers exposed to both benzene and insecticides).

Comparing to the unexposed workers, the exposed workers were reported to have increased risk in a variety of diseases. For example, in the original CAPM study, benzene-exposed workers experienced a total mortality risk of 2-fold when compared to unexposed workers. For all cancers, the mortality risks were 2.5-fold for exposed male workers and 1.8-fold for exposed female workers, when compared to their unexposed counterparts. Even in the expanded CAPM-

NCI study, some occupational groups (such as chemical or rubber manufacturers, painters or paint manufacturers) still exhibited significant increases from total mortality or all cancers (Li et al., 1994). It seemed unlikely that benzene was responsible for all these increases. No other studies have demonstrated such a broad mortality increase among benzene workers.

More specifically, increased mortality risks among exposed male workers when compared to unexposed workers were reported for many specific cancer sites, which have not been demonstrated to be related to benzene exposure in other studies; including lung cancer, liver cancer, stomach cancer, intestinal cancer, esophageal cancer, and nasopharyngeal cancer. For example, no study has ever demonstrated an association between benzene and nasopharyngeal cancer. These observations raised potential concerns regarding the comparability of the unexposed workers, potential under-ascertainment of cancer among the unexposed workers, and confounding exposures (or other risk factors) in the exposed group (Yin et al., 1989).

Cigarette smoking has been found to be a risk factor for leukemia (particularly AML) in a number of epidemiologic studies. The fact that lung cancer risk was significantly elevated (in men) and that a significant exposure-response relationship in terms of cumulative exposure to benzene was observed among the exposed workers in the Chinese benzene cohort called for an examination of the potential confounding by smoking (Yin et al., 1989 and 1994).

Based on case-control studies of leukemia in Shanghai, Lin et al. (1987, 1991) identified a number of significant risk factors: vinyl chloride (odds ratio, OR = 11.60), organic phosphate (OR = 2.15), toxic heavy metals (OR = 3.90), and radiation therapy or occupational exposure to radiation (OR = 6.58). The risk associated with the use of chloromycetin (chloramphenical) was also elevated (OR = 1.69, p = 0.06). In a large series of leukemia cases in the Henan Province, China, a large percentage of patients had a history of using chloromycetin (Wang et al., 1984). In the same series, the use of other medications was also reported. These potential confounding exposures or risk factors complicated the interpretation of findings (Yin et al., 1989 and 1994).

One of the areas of uncertainty in the Chinese benzene study involved the exposure estimates. Comparing the assumptions used in the development of estimates and the exposure estimates themselves to actual data reported identified a number of inconsistencies and/or inadequacy of exposure categories. For example, the actual average exposure of spray painters (full-day exposure) at a factory in Shanghai in 1965-1969 was 331 mg/m^3 (104 ppm), whereas the estimate developed by Dosemeci et al. (1994) for spray painters in the same time period was 22.1 ppm. There are other similar examples. It appeared that the exposure estimates were consistently lower than the actual exposure data. The "indirect validation" conducted by Dosemeci et al (1996), by definition, could not validate the absolute values of the estimates. Dosemeci et al. (1994) recognized that little confidence could be attached to the estimates: only 2% of the estimates for the time interval 1949-1959 and only 6% of the estimates prior to 1975 were rated in the "high confidence" category. Exposure-response analyses based on these exposure estimates should be interpreted with extreme caution.

Both CAPM and NCI have made substantial efforts in studying the relationship between benzene exposure and malignancies. The CAPM-NCI study is certainly one of the largest occupational studies. Because of the large number of workers who were exposed to relatively high levels of benzene (compared to, for example, levels at refineries in the US), data from China can provide further understanding of the health effects of benzene. Recent investigations of biomarkers will likely provide new insights as well (Rothman et al., 1996). However, for the results to be more reliable, the issues raised above regarding the data need to be addressed.

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Based on a review of the current benzene literature, considerations should be given to the following areas in future benzene epidemiologic research:

- exposure range in relation to study objective,
- exposure estimates for individual workers,
- adequacy of exposure categories,
- concomitant exposures to other chemicals,
- non-occupational risk factors,
- specific leukemia cell-types,
- other hematologic malignancies in addition to leukemia, and
- incorporating biomarkers or biomonitoring data in epidemiologic studies.

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ISSUES FOR DISCUSSION: BENZENE INDUCED LEUKEMIA-HUMAN STUDIES

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With respect to the Chinese cohort (Dosemeci et al., 1994; Hayes et al., 1996, 1997; Yin et al., 1987, 1989, 1994, 1996), the following issues are of concern: 1) What were the socioeconomic conditions prevalent in the members of the cohort i.e. factory workers and how are they different from those of benzene workers in the United States? Were there environmental factors i.e. diet, lifestyle habits, etc. of the Chinese cohort that were different from those of U.S. benzene workers studies (e.g. Rinsky et al., 1981, 1987) that could have a differentially adverse impact upon health conditions in these workers. How were they controlled for in the analysis? What were the known or potential carcinogens other than benzene in the workplace of the Chinese that could have produced confounding effects? 2) What was the evaluation and decision process used in each factory to assign estimated exposures to individual factory workers where no actual measurements were available? In the Dosemeci et al., 1994 Study, it is stated that 97% of the early estimates were not based upon actual measurements. What safeguards were employed to insure that the assigned exposures at the factory level were not biased but reflect the best judgement of the experts concerning potential exposures that existed at the time of the employment of the worker in each factory? 3) Other conditions, for which the risks were elevated, i.e. non-Hodgkin's lymphoma, myelodysplastic syndromes and hematologic neoplasms need to be confirmed in other studies. The latter two appear to be grouped categories containing an assortment of blood related disorders including cancer. Can the risk of individual unique conditions be determined?

Biomarkers of Leukemia Risk

Our main concern here is whether biological responses reported as biomarkers are really indicators of a population at a higher risk of leukemia (biomarkers of effect) or are they just indicative of a physiological response that may not be associated with a higher risk of cancer but perhaps only markers of exposure.

Insights from Petroleum Studies

With respect to petroleum worker studies, we have noted several observations. Most of these involve very low exposures to benzene, generally under 1 ppm. Most of them show little or no excess risk of leukemia, even ANLL. And, of course, there is little data addressing exposures between 1 ppm and 20 to 40 ppm (cumulative), a range that remains largely without information concerning sensitivity of risks (except perhaps the newer Chinese factory workers cohort). In the largest amalgam of petroleum workers studied thus far, Raabe and Wong (1996) studied a combined cohort of 208,000 workers (19 plants). The reported average exposure to benzene was found to be only 0.22 ppm. in such workers according to Runion (1988) based upon nearly 15,000 samples. The average cumulative exposure received if a worker remained employed in this industry for 40 years would be .22 ppm X 40 years or 8.8 ppm-years. More than likely, very few of the workers ever achieved a cumulative exposure of 200 ppm-years in this industry-wide study. The comparison background population that provided expected deaths in this study may have also sustained exposures that hovered around or close to this level of 0.22 ppm found in the "exposed" cohort. If this is so, then one would not expect to detect an increase in the risk of lung cancer in this study. As a matter of fact, the observed cases were not significantly different from expected..

An interesting theory was raised by Dr. Schnatter's 1996 study of petroleum workers (Schnatter et al., 1996), and that is, that peak exposures (excursions) may be more closely associated with the development of leukemia, rather than long term cumulative exposures. Dr. Schnatter used the median of the sets of exposure estimates of Rinsky, Crump and Paustenback to develop a new set of indices of exposure per person. An "average" total concentration per person was determined from the job category with

the greatest exposure (maximum) and of longest duration. This method made it possible to isolate subgroups with less exposure to specified concentrations of benzene and examine the relative risks to each of these subgroups. The idea was that these subgroups were unlikely to be exposed to concentrations greater than a specified concentration. Excursions and peak exposures could be potentially reduced in such groups and a better indication of what level of cumulative exposure is associated with an increased risk of leukemia could be ascertained. This assumes that it is the peak exposures that are responsible for the risk and eliminating them from the analysis would reduce any potential skewing of the results by the presence of peak exposures in the data. One difficulty with this idea is that the pliofilm cohort is not powerful enough at low doses to detect an elevated risk even if one were there. One possible data source that might be considered in the future for this type of analysis is the Chinese cohort data. Utilizing this data set might provide a large enough cohort at the lowest exposure levels that epidemiologists would no longer be plagued by small population sizes and consequently a lack of sensitivity at low exposures.

New Initiatives for Epidemiologic Research

An issue for discussion should be how a new epidemiology study would reduce uncertainties that remain in current epidemiology studies and by reducing them would help to improve the cancer risk assessment at low doses. Again, the cohort must be large, in order to detect significant risks at low doses of exposure to benzene. Secondly, it must exhibit a wide range of exposures to benzene, especially between 1 ppm and 50 ppm. and thirdly, it must be free of other confounding exposures as much as possible that could potentially influence the risk. The problems with exposure information are the biggest concern for risk assessors and incorporation of biochemical/ molecular biomarkers of exposure/ effects in mode of action. The estimated unit risk estimate from the Rinsky et al., 1987 study could change by as much as 1 or 2 orders of magnitude depending on which set of estimated exposures are used to derive the unit risk based on his data alone. If there were some method for deriving fairly reliable exposure estimates it would reduce the uncertainty of this parameter considerably.

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(The views expressed are that of the authors and do not necessarily represent EPA policy or endorsements)

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