



MEETING REPORT

**REPORT ON THE WORKSHOP ENTITLED:
 “MODELING CHEMICALLY INDUCED LEUKEMIA—IMPLICATIONS
 FOR BENZENE RISK ASSESSMENT”**

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Introduction

Benzene is a well established bone marrow toxin and human leukemogen. However, the health risk posed by exposure to benzene is a matter of some debate. A workshop organized by the University of California at Berkeley entitled “Modeling Chemically induced Leukemia—Implications for Benzene Risk Assessment” was held in Yountville, California on 11–13 February 1996 to discuss the molecular mechanisms of benzene toxicity and possibilities for modeling leukemogenesis. This workshop brought leading scientists in the fields of hematopoiesis, leukemia biology and benzene toxicology together with experts in mathematical modeling with the aim of building by consensus the foundations for a biologically based model that describes leukemogenesis and can be applied to risk assessment. This report summarizes key points of consensus and dissent on the above issues, and participants recommendations about the feasibility of biologically based modeling applied to benzene.

The format of the workshop emphasized discussion and consensus-building over formal presentations. For each topic area (benzene toxicology, leukemia biology and cancer modeling) there was an overview seminar followed by an extended discussion session including all

participants. Experts in the field acted as discussion leaders and questions posed in advance by the organizers served to focus discussions on key issues.

The general workshop aim was to evaluate the current state of knowledge of benzene-induced leukemia to determine whether and how the process can be modeled mathematically given what we know about benzene's bone marrow effects, leukemogenesis and available modeling techniques. We had set out to address the following questions:

- (1) What is the current state of knowledge of the mechanistic events that occur in benzene-induced leukemia?
- (2) What is the current state of knowledge and modeling of benzene toxicokinetics?
- (3) What is known about the toxic effects of benzene metabolites on hematopoietic cells, and which are critical to the leukemogenic process?
- (4) What options are available for modeling the dose–response relationships between benzene and/or metabolites and leukemia and/or other biological endpoints?
- (5) What are the best sources of data available to validate models of benzene toxicity and leukemogenicity and to determine parameters of such models?
- (6) What are the most critical gaps in data, knowledge and computing ability that limit modeling options, and how can they be addressed?

Abbreviations: CYP2E1, cytochrome P4502E1; GM-CSF, granulocyte macrophage colony stimulating factor; IL-3, interleukin 3; AML, acute myelogenous leukemia; FAC, Fanconi anemia C gene; IFN- γ , interferon gamma; MDS, myelodysplastic syndrome; TMBD, two mutation birth death; CFU-F, colony-forming unit fibroblast; MPO, myeloperoxidase; CFU-S, colony-forming unit spleen.

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Session I: Benzene Toxicology

In this session, chaired by Robert Snyder of Rutgers

University, three speakers presented current perspectives on benzene metabolism and toxic effects. Martyn Smith, from the University of California at Berkeley, presented an overview of benzene toxicity. He argued that it was likely that multiple metabolites are involved in benzene toxicity and that the quinone products of primary phenolic metabolites are the most critical. Benzene is metabolized in the liver, mainly via cytochrome P4502E1, to benzene oxide. The major subsequent oxidation product is phenol, which is either conjugated or further hydroxylated by CYP2E1 to hydroquinone [1]. Dr Smith presented evidence that individuals with a high rate of CYP2E1-mediated metabolism are at increased risk for benzene-induced hematotoxicity. NAD(P)H quinone oxidoreductase (NQO1; DT-diaphorase) is thought to play a role in detoxification of quinones. Recent evidence indicates that an increased risk of hematotoxicity is also observed in individuals who lack this enzyme, supporting the notion that quinone oxidation products formed from phenols and quinols are the ultimate toxic metabolites of benzene. Dr Smith described the types of genetic damage produced by phenolic metabolites of benzene and showed that these metabolites are capable of producing chromosome damage similar to that observed in workers exposed to benzene [2–4]. Dr Smith also discussed evidence that benzene and its metabolites produce oxidative DNA damage in bone marrow, which may also contribute to genetic changes in bone marrow cells [5].

Richard Irons, from the University of Colorado Health Sciences Center, discussed studies of benzene's epigenetic effects on hematopoietic cells. Hydroquinone increases the number of myeloid colonies that are produced in the presence of GM-CSF by IL-3-dependent CD34+ human bone marrow cells in clonogenic assays [6]. Increased clonogenicity occurs in the absence of noticeable cytotoxicity and results in a 1.5- to 5-fold increase in the number of cells in active cycle. Dr Irons proposed a model for the process of benzene-induced leukemia, in which this abnormal GM-CSF responsiveness of early progenitor cells is the initial step in benzene leukemogenesis in the bone marrow. In the context of the resulting abnormal hematopoiesis, genetic aberrations in myeloid progenitor cells would then occur, causing further deregulation and eventually culminating in acute myeloid leukemia (AML). To support the hypothesis, Dr Irons showed that other agents known or suspected to cause leukemia share with hydroquinone the ability to alter progenitor cell response to GM-CSF in his assay [7]. In contrast, there are several chemicals that cause aneuploidy which have not been associated with secondary AML, and additionally do not affect progenitor clonogenicity in the assay. This argues that genetic damage alone is not sufficient for leukemogenesis.

Michelle Medinsky from the Chemical Industry Institute of Toxicology presented a model of benzene dosimetry in mice. She suggested that previously reported non-linearities in the dose-response relationship between administered benzene and granulocyte count in mice [8] reflect underlying dose and dose-rate dependencies in formation of toxic metabolites [9]. Since both benzene and phenol can be hydroxylated by CYP2E1, competition between these substrates for the enzyme could explain some observed non-linearities in the formation of specific metabolites and thus in consequent toxic responses. Dr Medinsky's model predicts bone marrow concentrations of hydroquinone in mice that correlate closely with previously obtained data on femoral granulocyte depression after administration to mice of various doses of benzene. A key component of this model is the representation of heterogeneity in the spatial distribution of metabolic enzymes in the liver [10]. The model allows for the production and conjugation of phenol to occur in different liver subcompartments. Phenol and hydroquinone entering the liver will be immediately conjugated, whereas benzene will escape initial conjugation, passing through the liver to be activated in another zone. The phenol and hydroquinone produced from benzene are thus able to enter the circulation. This provides an explanation for the fact that phenol or hydroquinone exposures do not appear to be leukemogenic, yet exposure to the parent compound, benzene, is.

During discussion, David Eastmond, from the University of California at Riverside, raised the question of how to explain the latency of benzene-induced leukemia with laboratory data. The average latency for leukemia in Chinese workers exposed to benzene has been estimated at 11.4 years [11]. In order for toxic effects observed in the laboratory to contribute *in vivo* to the production of a malignancy that is expressed years later, the affected cell or its progeny must maintain self-renewal capacity throughout the latency period. Therefore, the life span and cycling behavior of cells targeted by benzene is of interest; only effects that can occur in long-lived cells are relevant to leukemogenesis. Another discussion topic was raised by Azra Raza from the Rush Cancer Institute concerning the presence of monoclonality in benzene-exposed patients. She argued that such data will be important in early identification of patients at risk for frank leukemia. Others, however, believe that hematopoiesis is monoclonal even in normal bone marrow and, therefore, that monoclonality in itself is not an indicator of abnormality.

Session II: Biology of Leukemia

During the second session, chaired by George Kalf, from Thomas Jefferson University, and Richard Larson,

from the University of Chicago, four participants spoke about the biology of leukemia and hematopoietic cells. This material provided a context for discussing the measured endpoints of benzene toxicity presented previously. Grover Bagby Jr, from the Oregon Health Sciences University, discussed evolution to AML in Fanconi anemia patients. He focused on the potential role of selective pressures acting on hematopoietic clones in the marrow. Suppression of FACC, the gene associated with the C complementation group of Fanconi anemia, using antisense strategies resulted in a reduction of the frequency of erythrocytic and granulocytic colonies in clonogenic assays [12]. This finding suggests a normal role for this gene in proliferation and differentiation of progenitor cells. Further data from mice lacking FAC suggest that myeloid progenitor cells from these mice have an abnormally high sensitivity to interferon-gamma (IFN- γ), resulting in high rates of apoptosis [13]. Dr Bagby proposed that hypersensitivity to IFN- γ could act as a selective pressure, resulting in clonal outgrowth of stem cells that have acquired resistance to its inhibitory effects. Clones that do not respond to inhibitory signals are potentially leukemogenic. This led to the question of how chemicals such as benzene could contribute to clonal selection, determining which clones ultimately grow out into frank leukemias.

Following Dr Bagby, Dr Raza spoke about the biology of myelodysplastic syndromes (MDS), emphasizing studies of bone marrow cell kinetics. She presented preliminary results of a new treatment regimen that was designed on the basis of a proposed model of MDS biology [14]. According to the model, an initial genetic aberration in a stem/progenitor cell confers a growth advantage and thus leads to monoclonal hematopoiesis. Complex interactions with positive and negative hematopoietic growth modulating molecules then induce extensive apoptosis in maturing cells, resulting in the cytopenias characteristic of MDS. Marrow cells in biopsy samples of MDS patients exhibit a simultaneous increase in the percentage of S-phase cells and in the rate of apoptosis when studied with a dual labeling method developed by Dr Raza and colleagues [15]. They propose that abnormal expression of cytokines such as transforming growth factor- β and tumor necrosis factor- α could be responsible. These cytokines have dual activity, suppressing cells at some stages of development and stimulating others. Dr Raza believes that monoclonal hematopoiesis is a prerequisite for the development of MDS, and it is in the context of monoclonality that further genetic aberrations associated with evolution of the disease arise. If this model is correct and monoclonality is an early 'step' toward malignant myeloid disease, then questions for benzene are how benzene produces monoclonality and whether

benzene can affect signaling by inhibitory growth factors.

Maria Pallavicini, from the University of California at San Francisco, discussed recent evidence that genetic aberrations present in AML at diagnosis are often detectable in cells in a stem cell compartment defined by CD34+ CD33- CD38- [16]. This finding suggests that the genetic events that are capable of causing leukemia to occur at earlier developmental stages than the predominant cell type observed in resulting leukemic clones. The implications for modeling are that kinetics and susceptibility to genetic damage of cells contained in a stem cell compartment must be considered in a mechanistic mathematical model, and the model should not conflict with developmental separation between the genetic insult and the ultimate leukemic cell type. Another interesting finding of this study was the observation that multiple genetically aberrant clones may be present in the stem cell compartment, including clones marked by aberrations that are not detected in the diagnosis karyotype. These data suggest that only some genetic abnormalities confer a proliferative advantage, resulting in leukemia *in vivo* or the ability to proliferate *in vitro*. Genomic instability can generate clones with additional abnormalities that may still derive from the same genetically aberrant stem cell. The prevalence of monoclonality in AMLs and preleukemic disorders resulting from benzene exposure is not known. Dr Pallavicini also explained the use of comparative genomic hybridization, a molecular cytogenetic technique that measures changes in DNA sequence copy number in tumor specimens. This methodology could be applied to leukemic or pre-leukemic specimens from benzene-exposed individuals to assess the extent of aneuploidy at various loci.

Dr Larson spoke about MDS and AML secondary to chemotherapy treatment for primary cancers. The factors that modulate susceptibility to therapy-related MDS or AML is, in Dr Larson's opinion, a critical outstanding issue in leukemogenesis. When compared to MDS cases, which are not associated with known chemical etiology, t-MDS patients have a much higher frequency of chromosomal aberrations; nearly all of these patients carrying aberrations show some involvement of chromosome 5 or 7 (e.g. refs [17, 18]). The definition of critical regions on chromosomes 5 and 7 has been an active area of research, but the genes involved in mediating the effects of deletion are still unknown [19, 20]. Patients with secondary MDS also have a high rate of progression to AML; if true for benzene-induced cases, this may have implications for developing a multistage model. Incidence rates of t-AML in treatment cohorts may reach a plateau with time, indicating that the latency period has an upper bound, e.g. ref. [21]. One case report was discussed in

detail by Dr Larson to illustrate that several different genetically altered clones can arise and die out during the time that the marrow is evolving from MDS to AML, suggesting that there is not always a simple monoclonal progression leading to malignant AML. Dr Larson also highlighted the different characteristics of t-AML secondary to alkylating agent therapy vs. therapy with inhibitors of topoisomerase II, which stimulated comparisons between the mechanism of action of these agents and benzene later in the meeting.

The panel discussion following these two presentations brought out some additional points. Dr Irons posed the question of whether the dysregulation of apoptosis observed in MDS patients is a cause or a result of their condition. Dr Kalf mentioned preliminary data from his laboratory showing that hydroquinone affects apoptosis of cultured hematopoietic cell lines, but has opposing effects at high and low doses. Phenolic metabolites of benzene have been shown, in Dr Ross's laboratory, to induce apoptosis in CD34+ human bone marrow progenitor cells [22]. Dr Kalf also raised the question of what role stromal tissue might play in the process of benzene-induced leukemogenesis. The importance of this question was supported by Troyce Jones from Oak Ridge National Laboratory who quoted from a paper by Donald Metcalf. The quotation addressed the fact that cytokines produced by stromal tissue play a critical role in controlling cell cycle and maturation behavior of hematopoietic progenitors, and that both normal and leukemic progenitors are cytokine-dependent. In an open discussion, Dr Eastmond pointed out that all known leukemogens cause myelotoxicity and induce chromosomal aberrations in peripheral blood lymphocytes. Therefore, it is reasonable, at this time, to propose that both myelotoxicity and genotoxicity contribute to benzene-induced leukemogenesis.

Session III: Modeling

Bernard Goldstein, from Rutgers University, and Suresh Moolgavkar, from the Fred Hutchison Cancer Research Center in Seattle, chaired the session on mathematical modeling of cancer and hematotoxicity. Dr Moolgavkar's seminar provided an introduction to carcinogenesis models, focusing on a Two-Mutation-Birth-Death (TMBD) model [23]. This model reflects a two-stage process in which stem cells are susceptible to transformation into an intermediate premalignant stage, followed by a second transformation resulting in malignant cells. The model provides advantages over other multistage cancer models in that it includes parameters describing proliferation and cell death in the normal and intermediate populations. This is particularly important for modeling cancer processes in highly dynamic tissue such as bone marrow. Under the

model, chemicals such as benzene can affect the rates of transformation to the intermediate and/or malignant stages, or kinetics parameters of these populations, or both. Dr Moolgavkar demonstrated the application of the model to epidemiological data on lung cancer and to experimental data concerning liver lesions in rodents. Advantages of biologically based cancer models include:

- (1) Complex problems can be broken up into constituent parts for analysis.
- (2) Model analyses generate testable research hypotheses.
- (3) Joint analyses of malignant and premalignant lesions can be done. If data relating exposure and pre-malignant lesions are available, estimation of the dose-response relationship can be based on data at lower doses than those that cause malignancy.
- (4) There is a wide family of incidence functions that can be used to look at time-to-tumor data, and time and age covariates are relatively easy to incorporate.

Dr Moolgavkar noted, though, that extrapolation to very low risks, such as is done for regulatory purposes, may be beyond the realm of science. Extremely low risk estimates that may be used as guidance in public health protection programs are essentially impossible to verify, and low-dose extrapolation from biologically based models is not necessarily any more accurate than extrapolation from curves generated using other methods.

L. Anthony Cox of Cox Associates spoke about ongoing efforts to model granulopoietic kinetics and hematotoxic effects of cyclophosphamide, a chemotherapeutic agent. He argued that TMBD models are appropriate for application to benzene-induced leukemia, but that basic model forms have to be extended in order to adequately describe benzene's effects on the bone marrow and certain aspects of leukemia biology. These include the feedback responses among hematopoietic cell populations, clonal hematopoiesis, induction of aneuploidy and transient synergy between hydroquinone and proliferation-promoting cytokines that has been observed *in vitro*. Dr Cox is optimistic that, using data currently available for benzene, a biological model can be developed that will be useful for low-dose risk prediction.

Dr Cox has extended and adapted a model of granulopoietic toxicity originally developed by Steinbach *et al.* for dogs [24]. The adapted model is able to predict decreases in human white blood cell counts that were observed after various regimens of cyclophosphamide administration. Dr Cox is applying his work

on cyclophosphamide modeling to benzene, with the goal of developing a risk model for benzene-induced leukemia. The benzene model consists of a set of submodels. First is a submodel describing the disposition of benzene in the body [25]. This provides input to genotoxicity and cytotoxicity submodels that yield a rate of transformation from a penultimate stage of leukemogenesis to AML and the number of cells in that stage, respectively. The hazard function for AML is then based on these two quantities. Dr Cox pointed out that there are quantitative data available for use in modeling concerning the relationship between benzene exposure concentration and a number of genetic and cytotoxic outcomes in rodents. However, since rodents are a poor model for benzene-induced leukemia, human data are needed. He suggested that further research is needed to understand quantitative relations between metabolite concentrations and cytotoxic responses, and how hematotoxicity is related to leukemia risk. The current model is not yet able to explain latency periods observed with chemically induced leukemia; this area also requires further work.

Dr Jones presented work in mathematical modeling of radiation-induced hematotoxicity. He discussed a model of cellular injury, death and repair after radiation exposure that was developed and tested based on data from 27 different animal experiments, performed in several different species [26]. The model suggests that mortality resulting from radiation damage to the bone marrow is more a function of damage to stromal cells than damage to the stem cell population. The model results predict experimentally determined survival curves for CFU-F populations well. This model has not been applied to benzene, but could mean that stromal cell toxicity at high doses is an important mechanistic consideration. Whether toxic effects in stem cells or stromal cells are most important in benzene hematotoxicity is not known. Dr Jones pointed out that, in general, overly complex mathematical models pose severe obstacles to users. If a model of benzene-induced leukemia were composed of several submodels, each depending on five or more parameters for description, the resulting overall model could prove too cumbersome to be useful.

During discussion, Frederic Bois, from Lawrence Berkeley National Laboratory, followed up on this comment by Dr Jones. He noted that, even if a biological model of benzene leukemogenesis can be constructed with consensus on the necessary parameters, validation of such a model is likely to be a formidable computational problem. Patrick Beatty from the Western States Petroleum Association commented that non-linearity of cytotoxicity endpoints in rodent studies occurs below saturation of metabolism, and therefore indicates an intrinsic lack of linearity for some toxic effects. He

stated that useful information for risk assessment can be obtained from a thorough review of benzene biology even if a complete biological model cannot be constructed. Dr Moolgavkar stated his impression that the database for benzene-induced leukemia might not be sufficient at this time for the construction of a useful biologically based model. He indicated that an important addition would be a large epidemiological data set that links benzene exposures to the disease endpoints of interest, AML and MDS. Such data would allow investigators to test model predictions of leukemia incidence, at least in the dose range encompassed by human data. Such data may be forthcoming from studies in China [27].

Alessandra Forni, from the University of Milan, gave a special seminar on the relationship between cytogenetically detected aberrations and leukemia. She has had extensive experience observing clinical correlates of benzene-induced hemopathies [28, 29]. In a recent analysis of chromosomal aberrations in peripheral blood lymphocytes and cancer risk, a correlation was found [30]. This raises the exciting possibility that a simple assay could be predictive of risk. However, the number of cases was small; without more data, a quantitative analysis of these data is not fruitful. If borne out by more data on leukemia, these results could open a new avenue for estimating human risk of exposure to benzene by measuring the frequency of genetic changes in exposed persons.

Session IV: Consensus and Directions for Future Research

The final sessions consisted of informal discussion and debate on points that had been raised during the workshop and on specific questions posed by workshop organizers. The following section of this report summarizes consensus, or near consensus, opinions and issues still outstanding. Some relevant background information is also included, as needed, to provide context for the consensus discussions.

The multistep process of benzene-induced leukemia

The participants agreed that benzene-induced leukemia is the product of a multistage process. During the course of the workshop, there was extensive discussion about effects induced by benzene or benzene metabolites observed in cultured cells, laboratory animals and humans. Which of these measured endpoints are critical to the leukemogenic process can still not be stated with certainty. Participants noted data gaps in several fields; these gaps precluded consensus in itemizing the stages of benzene leukemogenesis and identifying which stages are rate-limiting in the process. However, the workshop identified several factors considered most likely to play

a role in leukemogenesis by benzene. These include benzene toxicokinetics, dynamics of target cell populations in the bone marrow, genetic aberrations induced by benzene and non-heritable effects of benzene on bone marrow cells. Non-heritable effects of benzene are likely to play an important role in leukemogenesis by increasing the number of cells susceptible to genetic damage, altering hematopoietic regulatory signals or selecting for cells with abnormal characteristics.

Toxicokinetics

Benzene processing in the body involves several enzymatic and spontaneous steps which together produce oxidized metabolites that can be excreted in urine [1]. Primary metabolism of benzene in the liver generates phenol through a benzene oxide intermediate. Phenol can be conjugated for excretion, predominantly via sulfation in humans, or further hydroxylated to hydroquinone. Oxidation of both benzene and phenol is catalyzed by cytochrome P450 enzymes, primarily CYP2E1. Other metabolites produced include catechol and *trans,trans*-muconic acid. If hydroquinone and other phenolic metabolites reach the bone marrow before being conjugated, they can undergo secondary conversion by bone marrow myeloperoxidase (MPO) to highly toxic quinones and semiquinone radicals.

Most participants agreed that the phenolic metabolites, particularly hydroquinone, appear to be the critical compounds of interest in benzene-induced leukemia. Others believe that the ring-opened product *trans,trans*-muconaldehyde also plays a significant role. Accumulated data demonstrate that several metabolites may interact to produce the effects on target cells that are thought most likely to contribute to the leukemogenic process [31, 32]. However, Drs Snyder and Kalf believe that the data are insufficient to rule out an additional role for unmetabolized benzene. Oxygen radical production by secondary metabolism in the bone marrow was not addressed during this workshop, but may be pertinent to identifying the ultimate leukemogenic agent(s). Despite years of research, definition of the critical leukemogenic species needs further resolution.

Several efforts to apply mathematical modeling to benzene distribution and metabolism have been undertaken [33–35], and two investigators who have contributed extensively in this area, Drs Bois and Medinsky, were present at the workshop. These models represent the first components of a comprehensive mechanistic model of benzene-induced leukemia. Medinsky's model, developed using data from exposed rodents, is able to predict levels of hydroquinone production from a range of exposures that correlate well with data on bone marrow toxicity in mice. Animal models are useful precursors to human models; however, parameters derived from analysis of animal data have not yet been

validated for application to human exposures. A model of benzene distribution and phenol excretion in humans has been developed by Dr Bois [36]. The model was statistically fitted to data using a population pharmacokinetic approach and Bayesian numerical techniques, and has been validated with data from controlled human exposure [37]. However, this model has not yet been tested for the ability to predict data to which it was not fitted. Several toxicokinetic studies have identified an extensive variability in metabolic activity in human populations and between species and strains of laboratory animals. More work is needed in the area of model validation before the production and distribution of individual benzene metabolites in a heterogeneous human population can be accurately predicted from exposure data.

Dr Bois' model predicts that the fraction of benzene converted in primary metabolism to total metabolites in humans is linear below continuous exposure concentrations of 10 ppm in the air. However, work by Medinsky's group suggests that the fractions of total metabolites contributed by phenol and hydroquinone are likely to vary with exposure. One possible source of non-linearity in production of specific metabolites is competition between benzene and phenol for CYP2E1. Production of secondary metabolites from CYP2E1 oxidation of phenol occurs more rapidly at low than at high doses because there is less benzene in the liver to compete for metabolism. According to this model, the rate of production of hydroquinone at benzene exposure concentrations up to 100 ppm yields a supralinear internal dose curve. At higher doses, phenol is less likely to be oxidized; thus, phenol conjugates predominate over secondary phenolic metabolites. This result has important implications for the dose–response relation between benzene exposure and toxic effects that are caused by hydroquinone.

Target cells

To analyze mechanisms of leukemogenesis and model the dynamics of the process it is critical to define which cell populations are affected by benzene exposure and must be included in an acceptable model. Benzene has been shown to affect a number of different cell types in exposed humans and under various experimental conditions; the question to be resolved is which effects in which cells lead to leukemia.

Genetic aberrations in AML can be detected in CD34+ CD33– CD38– stem cells [16]. This suggests that the ancestral leukemic cell in these patients resides in an immature subset of the CD34+ cell population and that, to produce leukemia, genetic alterations must occur at an early developmental stage. For benzene, there are no data that directly address which bone marrow cells are targets for genetic damage in humans. Published

experimental data indicate that benzene can produce chromosome damage in rodent erythrocytic precursors, cultured lymphocytes and various hematopoietic cell lines [38]. In exposed humans, increased frequencies of genetic aberrations have been observed in peripheral cells of lymphocytic and erythrocytic lineages [38], but the cells in which damage originally occurred have not been identified. CD34+ cells express myeloperoxidase which plays a role in generating toxic benzene derivatives [39]. Available evidence is, therefore, consistent with a hypothesis that benzene is able to target CD34+ hematopoietic cells for genetic damage, and based on the observations in AML patients, immature sub-compartments of this population may be the key targets.

CD34+ bone marrow cells are also important targets for epigenetic effects of benzene. The ability of hydroquinone to increase clonogenicity induced by GM-CSF in culture, noted by Dr Irons and colleagues, acts on CD34+ cells. Further, this effect of benzene is thought to act primarily on early stage CD34+ cells that should not yet be sensitive to GM-CSF. Based on the above evidence, workshop participants agreed that CD34+ cells undergo alterations in genetics and behavior in response to benzene exposure and therefore should be considered a key target cell population in a mechanistic model.

Benzene-induced myelotoxicity has been observed in progenitor and precursor cells in rodents, ranging from CFU-S to erythrocytic precursors [38]. Toxicity to compartments later in the developmental hierarchy may alter the kinetics of less mature compartments through feedback mechanisms. Therefore, inclusion of population kinetics of more mature hematopoietic cells affected by benzene exposure may be important for modeling leukemogenesis that is initiated in stem cells.

Toxicity and other epigenetic effects of benzene have also been observed in cells of the hematopoietic stroma. What role these effects play in leukemogenesis is not clear. Under conditions of repeated benzene exposure, the ability of the stroma to carry out support and regulatory functions, such as cytokine secretion, may be compromised. No consensus opinion was reached as to whether stromal damage is a necessary component of benzene-induced leukemogenesis.

Role of genetic damage in benzene-induced leukemia

The frequency of clonal chromosomal abnormalities in MDS and AML can approach 100% of patients, depending on the population under study. This, together with accumulating evidence that benzene is able to cause the relevant types of genetic damage, led participants to the consensus that production of genetic abnormalities is an essential component of benzene-induced leukemia. It is reasonable to hypothesize that

leukemogenesis involves genetic alterations in multipotential stem cells or early myeloid cells, but there was not consensus about whether such changes are required to initiate leukemogenesis, occur after other initiating events, or both.

Benzene and its metabolites produce numerical and structural chromosomal damage in exposed humans, laboratory animals and cultured human cells. The types of damage that have been observed are consistent with the genetic changes that are identified in MDS and AML patients. Loss of chromosome 5 or 7 is the most common genetic aberration in secondary MDS/AML, and an increased frequency of aneusomies was detected in peripheral lymphocytes from workers exposed to benzene [3]. These results are supported by findings of induced aneuploidy in experimental systems [2, 40]. Benzene has been implicated in disruption of the mitotic spindle [41], which could result in non-dysjunctional loss and gain of chromosomes in daughter cells.

Interstitial deletions and stable rearrangements such as balanced translocations are also common karyotypic findings in MDS and AML. As yet, however there is no strong evidence that links benzene exposure to the specific translocations, inversions and other rearrangements that predominate in AML. Unstable and stable chromosome aberrations in the peripheral blood of patients with benzene-induced hemopathy were reported by Dr Forni and colleagues [28, 29]. Benzene metabolites are clastogenic in various assays, causing DNA strand breaks. Deletions and translocations could result from aberrant religation of strand breaks by cellular enzymes during repair processes. Workers exposed to benzene have an increased prevalence of red blood cells expressing the products of mitotic recombination events at the glycophorin A locus [4]. These require interchromosomal interaction to occur and thus may be marker events for other chromosomal rearrangements.

It has been known for some time that benzene metabolites can produce adducts with cellular macromolecules, both proteins and nucleic acids, but benzene is negative or only weakly positive when tested in standard mutation assays [42]. It is still unclear as to what role DNA adduct formation plays in benzene toxicity; workshop participants placed little emphasis on this potential mechanism. However, the formation of adducts with cellular proteins may be a clue to the mechanism that underlies the above forms of benzene-induced genetic damage. Disruption of proteins with key roles in maintaining genome integrity, such as histones, topoisomerases, repair enzymes or microtubules, may be a critical part of benzene leukemogenesis [43].

Benzene-induced hematotoxicity

There was considerable discussion about the prevalence of hematologic abnormalities prior to AML in

benzene-exposed patients. It is clear that hematotoxicity does not necessarily lead to leukemia; there are many documented cases of recovery from benzene-induced bone marrow suppression without subsequent leukemia [44]. However, whether benzene-induced leukemia can occur without prior toxicity is a matter of debate. Among published cases of benzene-induced leukemia, there are no examples of patients who developed AML without any evidence of prior hematotoxicity. However, published data will be biased, since hematologic work-up is less likely to be done during the pre-leukemic phase for subjects who do not have symptoms preceding their AML diagnosis. There was a consensus opinion among a majority of workshop participants that hematotoxicity, as evidenced by an overt decrease in white blood cell count, often precedes benzene-induced leukemia and can play a role in disease causation, but is insufficient alone to predict leukemia. There was not consensus on whether hematotoxicity is an obligatory prerequisite.

Two mechanisms by which toxicity to hematopoietic populations could increase leukemia risk were discussed. If benzene's genetic effects target dividing cells, then increased proliferation to compensate for the loss of cells to toxicity would result concomitantly in greater numbers of cells susceptible to genetic damage. Alternatively, the key result of increasing the size of an actively dividing population might be to increase the probability that a previously acquired genetic alteration comes to be expressed in an actively dividing cell.

Indirect effects on hematopoietic cells mediated by the hematopoietic microenvironment were also considered. As mentioned previously, benzene metabolites are toxic to stromal cells in culture. Since these cells are important sources of cytokines, stromal toxicity *in vivo* could cause alterations in the physiological feedback and signaling pathways that are necessary for normal hematopoietic regulation.

Role of apoptosis in benzene-induced leukemia

The role of apoptosis in benzene-induced leukemia, and leukemogenesis in general, is a matter of debate. However, cell death rates are key parameters in some quantitative carcinogenesis models and so it is important, from a modeling perspective, to understand how apoptosis is altered by exposure. Hydroquinone, catechol and benzenetriol cause apoptotic cell death in CD34+ human bone marrow progenitors and other cultured cells [22]. Significant induced cell death in the marrow of exposed individuals may result in abnormal bone marrow kinetics of the progenitors that escape apoptosis. This presumably would be an early response to exposure, and could contribute to leukemogenesis in the same manner discussed above. Further, induction of apoptosis in bone marrow cells could contribute to the

selection of aberrant clones of progenitor cells. Genetic alterations that confer resistance to benzene-induced cell death may be selected for under chronic exposure conditions. Some participants thought that genetic blocks to apoptosis are likely to play a role late in leukemogenesis, associated with a progression from MDS to AML.

Other epigenetic events

The number of cycling stem or progenitor cells may be altered by transient mechanisms other than cell death, such as the increased clonogenicity in response to GM-CSF reported by Dr Irons and colleagues. These investigators propose that synergy of hydroquinone with other cytokines recruits quiescent CD34+ cells into active cell cycle. As stated above, an increased number of dividing granulocytic progenitors may result in a larger target population for genetic damage. Direct stimulation of cell proliferation by benzene metabolites, secondary metabolites, or oxygen radicals are other possible mechanisms for increasing the number of target CD34+ cells. Some participants expressed skepticism that benzene is likely to act via this classic 'tumor promoter' mechanism; however, this possibility has not been formally ruled out.

Benzene metabolites alter the function of stromal macrophages [45–47]. Production of processed IL-1 by macrophages is reduced in the presence of benzene metabolites; if true *in vivo*, this may indirectly affect hematopoietic cells and thus contribute to leukemia.

Benzene and clonal selection

The participants concluded that clonal selection is an important event in leukemogenesis. It was suggested that benzene or its metabolites could exert some selective pressure on hematopoietic cells that supports or allows outgrowth of genetically altered clones. What kind of selective force could be created by benzene exposure is not clear; however, the mechanisms discussed above may contribute. It could be speculated that chronic exposure selects for resistance to benzene-induced toxicity. Further, if genetic alterations arise that confer resistance to programmed cell death, then induction of apoptosis could be a mechanism for the selection of aberrant clones of progenitor cells. Benzene exposure could also transiently disrupt the signaling pathways required for growth regulation through epigenetic action on the bone marrow. Chronic or repeated exposure could create an abnormal marrow environment in which leukemic clones arise or survive more effectively.

Thus, there are several mechanisms of toxicity, acting at the cell or tissue level, through which benzene may promote leukemogenesis. What is not known is which of these are critical for disease production. Much more

work is needed to distinguish, first, which of these events are actually occurring in humans exposed to benzene, and second, which of these events are able to increase the risk of AML.

Dose-response issues

There was consensus that benzene induces leukemia via a number of different mechanisms that result in both chromosomal damage and other alterations of hematopoietic cells. Due to this complexity, modeling the relationship between the exposure concentration of benzene and the probability of AML in a population using a mechanistic approach is a difficult task. The shape of the dose-response curve for leukemia induction will be influenced by the set of underlying dose-response relations between benzene and each of the several toxic effects that together produce AML. Dose-response curves are not expected to be the same for all effects. Understanding how each effect varies with exposure concentration, and with other parameters of exposure such as duration and frequency, is necessary if the effects are to be included explicitly in a model. Currently, there is a significant amount of uncertainty for some of the potentially critical cell or molecular responses.

Also, participants concurred that the predominant metabolites and therefore the predominant toxic effects of benzene are likely to vary with exposure. For example, hydroquinone-mediated genetic damage may be an important effect at low dose, while cytotoxicity may predominate at higher doses. This possibility, together with evidence that there are likely to be multiple mechanistic pathways for leukemogenesis, suggests that the set of events that are produced may vary also with exposure parameters. If true, this complicates mechanistic dose-response modeling considerably.

Quantitative dose-response data are available for some genetic effects in cultured cells, and for clastogenicity in rodents. However, a primary issue in dose-response estimation for human leukemia is the difficulty in extrapolating doses used in these experiments to the concentrations in environmental media to which humans are likely to be exposed. Further, many laboratory results are generated with a single acute dose, whereas the interest for human health risk is primarily in chronic exposure scenarios. Pharmacokinetic models may be useful extrapolation tools, but precise estimates of metabolite concentrations in human and rodent bone marrow after various exposures are still not available, causing considerable uncertainty. Gain or loss of chromosomes by microtubule-mediated non-dysjunction in target cells was also discussed. Based on theoretical arguments, it was concluded that the dose-response relation is likely to be non-linear, with the possibility of

a threshold. Disruption of microtubules is, however, just one possible mechanism of aneuploidy induction. Determining a dose-response relationship for overall genetic damage will remain a complex problem, since there are multiple mechanisms by which benzene produces genetic abnormalities in target cells, and each mechanism may have a different relation with dose and dose-rate parameters.

Another issue complicating dose-response assessment is that background levels of some benzene metabolites in human bone marrow are likely to be high, based on data from rodent species and humans. There was disagreement about whether high background levels of leukemogenic metabolites are likely to increase or decrease the risks of low exposure to benzene over that which would be calculated without consideration of background levels. The quantitative characteristics of leukemogenic response to exposure at low doses of benzene is being discussed by participants, and the results of these discussions will be reported elsewhere. However, there is not likely to be a consensus, and from the above discussion, it is clear that the shape of the dose-response curve in the low-dose region can only be guessed. Whether mechanistic modeling will help to refine the guesswork for extrapolated doses is not clear at this time.

Modeling issues

The foregoing discussions indicate significant complexity, uncertainty and lack of consensus concerning precise mechanistic details of benzene-induced leukemia. Most participants considered that current uncertainties limit the ability of modeling to explicitly consider all relevant mechanisms, such as the formation of several types of genetic aberrations, disruption of proliferation, differentiation or apoptotic behaviors through genetic change or epigenetic chemical interference, and the extremely complex and subtle regulation of hematopoietic processes under normal feedback systems. The workshop concluded that an ideal future model would be able to describe quantitatively benzene pharmacokinetics in humans, relate dose measures to the above pharmacodynamic mechanisms and account for observed epidemiologic features of benzene-induced leukemia, such as patterns of latency and susceptibility. It was also agreed that, when they are to be used for public health protection, mathematical models should be developed using measurable quantities to the extent possible. Therefore, participants felt that, while further measured data are generated, modeling efforts should be directed at developing simpler models and applying them first to data from other sources.

General concepts of the TMBD model, as described above, could be used as a guide for a simplified leukemia modeling approach. To apply the model, the

target cell population that ultimately contains ancestral leukemic cells and a representative intermediate stage of leukemogenesis would have to be defined. The complex regulation of hematopoiesis would be reduced to a limited number of dose-sensitive parameters describing rates of cell division, death and maturation. Other parameters would describe how the rates of transformation from normal to intermediate and intermediate to malignant cells vary with dose. Estimates of internal metabolite concentrations derived from previous pharmacokinetic modeling could provide input for a TMBD leukemia model.

CD34+ progenitors are candidates for the initial target cell population. However, this is a heterogeneous population; rates of cell division, differentiation and death that are required for application of the model may be difficult to define. In addition, the number of CD34+ cells fluctuates in normal bone marrow, further complicating population dynamics. Some quantitative data on cell numbers and dynamics are available for hematopoietic stem cells in animals. The models of hematotoxicity presented by Drs Cox and Jones are an important step toward mathematical description of stem cell population dynamics. However, stem cell kinetics for humans are not well understood, and application of the models to humans will require extensive validation.

Workshop participants explored at some length the possibility that MDS could represent an intermediate stage on the pathway to AML in a preliminary TMBD model. Description of biological events leading to MDS would then be condensed into a few parameters linking exposure or some other dose metric to the probability of MDS. In the second stage, the population kinetics and rate of transition to AML that occurs in MDS cell clones would be modeled. Some participants were skeptical about whether cellular dynamics and the dose/rate relationships controlling chemically induced transition from a normal to dysplastic and dysplastic to leukemic cell can be reasonably represented by a limited number of parameters. Another difficulty with this approach is that the transition from MDS to AML might be best characterized as cancer progression rather than as a stage in the genesis of malignancy; the TMBD model was originally conceived to describe an abnormal, yet benign intermediate stage. In fact, MDS is also a lethal disease that might be best considered part of the end stage. Additionally, while epidemiologic studies implicate benzene in causing both MDS and AML, there is still debate about whether AML is always preceded by MDS in benzene-exposed patients.

Dr Raza suggested that, alternatively, establishment of monoclonal hematopoiesis by an abnormal stem cell could be modeled as the intermediate stage. This would precede clinical MDS or AML, which could be combined into the malignant population. While this

approach is theoretically appealing, a difficulty from a modeling perspective is the lack of quantitative data addressing the timing and frequency of abnormal clonal dominance in individuals who do not yet have MDS or leukemia. Data from benzene-exposed cohorts would be a particularly important contribution to the data currently available for analysis of benzene-induced leukemia.

In order for any mechanistic model of leukemogenesis to be validated, it must be applied to existing data that relate known human exposures to the probability of contracting MDS/AML. While there are epidemiologic data of this sort for benzene, estimation of exposure is a complex task, with considerable uncertainty. Therefore, a suggested approach was first to develop a biologically based risk model for chemotherapy-related AML (t-AML). Such a model then could be applied to data from a benzene-exposed cohort, to see if parametric estimates, derived from a study of t-AML, are appropriate for the case of benzene exposure. Dr Cox's work on modeling cyclophosphamide is a step in this direction. In terms of longer latency, frequent preceding dysplastic phase and common aneusomies, benzene-induced leukemia is more akin to leukemia following therapy with alkylating agents than to AML secondary to other exposures. Other advantages of modeling t-AML as compared to benzene-AML are more precise dose information, comprehensive hematological data over time and precise estimates of time-to-cancer in these patients. A drawback, considered by participants, is that therapeutic agents have more potent myelosuppressive capability than benzene/metabolites and are given in high doses; thus, bone marrow biology may be significantly different in these patients compared to that resulting from low-dose benzene exposure. This, in turn, may mean that leukemogenesis is a qualitatively different process in these patients. Another possible drawback raised is that mortality due to primary tumors modifies the number of leukemia deaths observed in chemotherapy cohorts.

A final issue that has to be considered in modeling benzene-induced leukemia in the general population is that there is considerable interindividual variability that may influence risk. Some of the genetic factors important in metabolic variability are becoming known, but other aspects of susceptibility are less well characterized. For example, the factors controlling whether patients who suffer benzene-induced myelosuppression progress to AML or recover after exposure is reduced or removed are unknown. The extent to which susceptibility factors will dictate leukemia risk and the extent to which leukemia is a manifestation of stochastic processes are not known.

Data gaps and future research recommendations

Data insufficiencies in several fields were identified, during the workshop, that pose obstacles to understanding the mechanisms of benzene-induced leukemia. Some of these have been mentioned above. A panel of participants convened at the end of the meeting to highlight categories of information needed and to compose a list of future research recommendations.

Several classes of data on humans exposed to benzene are needed. More extensive epidemiological data, with good exposure estimation, are necessary to allow model predictions to be tested and validated. Further, data on preleukemic hematology of benzene-exposed persons, such as abnormal monoclonality and blood cell counts would make a significant contribution. Specific measures of early genetic damage in humans with known exposure to benzene would help define the biological events between exposure and disease by providing internal markers. This may be useful in risk prediction and aid in identification of the steps and stages in benzene-induced leukemia. Epidemiologic and genetic biomarker data soon may be forthcoming when further studies from a large cohort of workers in China are published by investigators from the National Cancer Institute, the Chinese Academy of Preventive Medicine and the University of California at Berkeley [4, 27]. In addition, physicians examining patients exposed to benzene, or with hematologic abnormalities that could be due to benzene exposure are urged to contribute in these areas.

There is a need to validate further toxicokinetic models and to assess metabolic susceptibility factors using human data. Collection of relevant information for this is problematic as it involves exposure of human volunteers to an established carcinogen. However, data collected in the Chinese cohort study on urinary metabolites of benzene and *in vitro* studies of cell-specific metabolism and toxicity in defined human bone marrow cell populations may be useful.

Continued basic research in hematopoiesis and leukemia biology is critical. Issues include questions about the cell population that contains targets for leukemic transformation, such as cell numbers and division rates, quiescence patterns, maturation regulation and apoptotic behavior. Further understanding of the phenotypic consequences of common genetic aberrations in MDS and AML is also needed to assist in identifying the stages of leukemic transformation. There remain many questions concerning the roles played in leukemogenesis by general hematotoxicity, induction of apoptosis and epigenetic disruption of physiological regulation. Also, factors that influence susceptibility to chemically induced leukemia or to leukemia subsequent to hematotoxicity are important for estimating population risk.

The panel outlined a four-point approach for advancing toward a mathematical description of benzene-induced leukemia:

- (1) Develop a preliminary mechanistically based descriptive model of treatment-related leukemia; attempt quantitation and validation using data from chemotherapy cohorts.
- (2) Determine whether benzene is sufficiently similar to treatment-related leukemia, or of a subset of treatment-related leukemia, that the derived model can be appropriately generalized to benzene. Data from the NCI-CAPM Chinese worker cohort and/or animal studies can be used for comparison of leukemogenic mechanisms between benzene and chemotherapy compounds.
- (3) Determine the metabolic pathways and toxicokinetic models that are specifically pertinent to benzene leukemogenesis, bearing in mind that the relationships between specific metabolites and the relevant toxic mechanisms for leukemogenesis are still being explored.
- (4) Ultimately, use a variety of modeling approaches to develop mathematical relationships between benzene exposure and leukemia and validate these with epidemiological data.

Workshop participants:

Dr Grover Bagby Oregon Health Sciences University	Dr Patrick Beatty Chevron Research and Technology
Dr Frederic Bois Lawrence Berkeley National Laboratory	Dr L. Anthony Cox, Jr Cox Associates
Dr David Eastmond University of California, Riverside	Dr Alessandra Forni Università Degli Studi Di Milano
Dr Donald Gaver Naval Postgraduate School	Dr Bernard Goldstein Rutgers University
Dr Rogene F. Henderson The Lovelace Institutes	Dr Richard Irons University of Colorado
Dr Troyce Jones Oak Ridge National Laboratory	Dr George Kalf Jefferson Medical College
Dr Richard Larson University of Chicago	Dr Michele A. Medinsky Chemical Industry Institute of Technology
Dr Suresh Moolgavkar Fred Hutchinson Cancer Research Center	Dr Maria Pallavicini University of California, San Francisco

Dr Azra Raza Rush Presbyterian-St. Lukes Medical Center	Dr David Ross University of Colorado	10.35-11.10 am 11.10-12.00 pm	Secondary Leukemias: R. Larson Discussion led by D. Eastmond, M. Smith, B. Goldstein, A. Forni, T. Cox
Dr Martyn Smith University of California, Berkeley	Dr Robert Snyder Rutgers University	12.00-1.00 pm Afternoon	Lunch Mathematical Modeling Session Chairs: B. Goldstein, S. Moolgavkar
Dr Lauren Zeise California Environmental Protection Agency	Dr Luoping Zhang University of California, Berkeley	1.00-1.40 pm 1.40-2.15 pm	Overview of Carcinogenesis Models: S. Moolgavkar A model of Benzene Hematotoxicity: L. A. Cox
Observers:		2.15-2.50 pm	Lessons from Modeling the Effects of Radiation: T. Jones
Dr Jennifer Galvin Phillips Petroleum Company	Dr Charles Lapin ARCO	2.50-3.15 pm 3.15-3.55 pm	Break Discussion led by F. Bois, D. Gaver, A. Raza, L. Zeise, R. Irons, G. Bagby, M. Medinsky
Dr Mary Paxton American Petroleum Institute	Dr F. Benjamin Thomas Compliance Systems Inc.		

Dr John Budroe California Environmental Protection Agency	Dr Thomas A. McDonald California Environmental Protection Agency
Dr David Ting California Environmental Protection Agency	Ms Elinor Fanning University of California, Berkeley
Mr Joe Wiemels University of California, Berkeley	

Workshop Program

Sunday 11 February

Lunch Reception

12.00-2.00 pm	Introductory Remarks Goals of the Workshop: M. Smith NIEHS Perspective: W. Suk Cal EPA Perspective: L. Zeise Industry Perspective: P. Beatty
Afternoon	Benzene Toxicology Session Chairs: R. Snyder, M. Smith
2.00-2.40 pm	Overview of Benzene Toxicology: M. Smith
2.40-3.15 pm	Altered Differentiation in Benzene Toxicity: R. Irons
3.15-4.00 pm	Discussion led by R. Snyder, D. Eastmond, A. Forni, B. Goldstein
4.00-4.15 pm	Break
4.15-4.50 pm	Pharmacokinetics of Benzene: M. Medinsky
4.50-5.30 pm	Discussion led by F. Bois, G. Kalf, D. Ross, R. Henderson

Monday 12 February

Morning	Biology of Leukemia Session Chairs: G. Kalf, R. Larson
8.00-8.35 am	Hematotoxicity and Leukemia: G. Bagby
8.35-9.10 am	Apoptosis and Leukemia: A. Raza
9.10-9.40 am	Discussion led by R. Irons, G. Kalf, T. Jones, D. Ross
9.40-10.00 am	Break
10.00-10.35 am	Genetic Changes in Leukemia: M. Pallavicini

Tuesday 13 February

Morning	Special Lecture Chair: M. Smith
8.00-8.35 am	Chromosome Abberations as Predictors of Risk from Benzene Exposure: A. Forni
8.35-9.00 am	Discussion led by B. Goldstein, R. Larson, M. Pallavicini, F. Bois, L. Zhang
	Group Consensus Consensus Meeting I
9.00-10.10 am	Break, Informal Discussion
10.10-10.30 am	Consensus Meeting II
10.30-12.00 pm	Lunch-Closing Remarks
12.00-1.30 pm	
Afternoon	Write-up (organizers and invitees only)
1.30-5.00 pm	Write-up Participants from U.C. Berkeley, CalEPA, Industry and selected invitees will work together to write up the proceedings of the workshop, to create a meeting report that summarizes the key conclusions that have emerged. Points of consensus and key points of dissent will be included.

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