Biomarkers of Leukemia Risk: Benzene as a Model

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Although relatively rare, leukemias place a considerable financial burden on society and cause psychologic trauma to many families. Leukemia is the most common cancer in children. The causes of leukemia in adults and children are largely unknown, but occupational and environmental factors are strongly suspected. Genetic predisposition may also play a major role. Our aim is to use molecular epidemiology and toxicology to find the causes of leukemia and develop biomarkers of leukemia risk. We have studied benzene as a model chemical leukemogen, and we have identified risk factors for susceptibility to benzene toxicity. Numerous studies have associated exposure to benzene with increased levels of chromosome aberrations in circulating lymphocytes of exposed workers. Increased levels of chromosome aberrations have, in turn, been correlated with a heightened risk of cancer, especially for hematologic malignancy, in two recent cohort studies in Europe. Conventional chromosome analysis is laborious, however, and requires highly trained personnel. Further, it lacks statistical power, as only a small number of cells can be examined. The recently developed fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR)-based technologies have allowed the detection of specific chromosome aberrations. These techniques are far less time consuming and are more sensitive than classical chromosomal analysis. Because leukemias commonly show a variety of specific chromosome aberrations, detection of these aberrations by FISH and PCR in peripheral blood may provide improved biomarkers of leukemia risk. — *Environ Health Perspect* 106(Suppl 4):937–946 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-4/937-946smith/abstract.html*

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Classification of Leukemia

Leukemia is a cancer of the blood-forming system and has been defined as the uncontrolled proliferation of hematopoietic cells that have lost the capacity to differentiate normally to mature blood cells (*1*). Leukemias are generally classified into lymphocytic and myeloid categories, according to the cell lineage affected, and can be further designated as acute or chronic leukemias. Acute leukemia is characterized by aggressively proliferating cells that rapidly colonize the bone marrow and prevent normal blood cell maturation; chronic leukemia progresses much more slowly. Thus, there are four general categories of leukemias: acute lymphocytic (ALL), chronic lymphocytic (CLL), acute myeloid (AML), and chronic myeloid (CML). These classifications are not exhaustive because a small number of acute leukemias have features characteristic of both the myeloid and lymphoid lineages, and are thus designated acute biphenotypic leukemias (*2*). In addition, another minor form of chronic leukemia, hairy cell, accounts for less than 2% of all cases (*3*).

The frequencies of these four major types of leukemia in children differ from those in adults (Table 1). Based on a research report from the National Cancer Institute (NCI), AML is the most common form among adults, followed by CLL (*3*). On the other hand, ALL, which occurs relatively rarely among adults (approximately 6%), accounts for most childhood leukemia cases (*4*). The acute leukemias are further classified into subtypes under the French–American–British (FAB) system. The subtypes and frequencies of acute leukemias are summarized in Table 2. In the United States, myeloblastic and/or monoblastic (M1, M2, M4, M5) leukemias are the most common AMLs in both children and adults (*4,5*), with the M4 category being the most common form in newborns (*6*). L1 is the most common subtype of ALL in children, whereas L2 is more frequently seen among adults (Table 2) (*7*). However, the FAB classifications have not proved useful in the clinical management of ALL; therefore, ALL is more often subclassified according to immunophenotype in the clinical setting (*4*). Under this system, early pre-B cell and pre-B cell are the most common forms of childhood ALL (Table 2).

Incidence of Leukemia

The global incidence of leukemias is about 8 to 9 per 100,000 people each year. Approximately 250,000 new cases occur annually worldwide, about 28,000 of those in the United States (*8,9*). Leukemia accounts for 2.5% of overall cancer

Table 1. Classification of leukemia and frequencies in children and adults.

Cell lineage	Frequency, % Childhood	Adult
Lymphocytic		
ALL	75	6
CLL	Rare	25
Myeloid		
AML	20	54
CML	5	15

Data from the National Institutes of Health (*3*).

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Abbreviations used: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; BT, 1,2,4-benzenetriol; CAPM, Chinese Academy of Preventive Medicine; CAT, catechol; CI, confidence interval; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CYP2E1, cytochrome P4502E1; del(5q), long-arm deletion of chromosome 5; del(7q), long-arm deletion of chromosome 7; EMF, electromagnetic fields; FAB, French–American–British; FISH, fluorescence *in situ* hybridization; GPA, glycophorin A; GST, glutathione *S*-transferase; GSTM1, glutathione *S*-transferase μ; GSTT1, glutathione *S*-transferase θ; HQ, hydroquinone; MDS, myelodysplastic syndromes; MPO, myeloperoxidase; NCI, National Cancer Institute; NQO1, NAD(P)H:quinone oxidoreductase; PCR, polymerase chain reaction; RT, reverse transcriptase; t-AML, therapy-related acute myeloid leukemia; TWA, time-weighted average.

incidence and 3.5% of cancer mortality in the United States. However, its incidence among children demonstrates its significance. Although childhood cases (through 14 years of age) account for about 12% of all leukemias, childhood cancer is the second biggest killer of children (after accidents) and leukemia is the most common form of childhood cancer (*10*). The incidence of childhood ALL in the United States has increased approximately 20% over the past two decades, mostly in the 0- to 4-year-old age group (*10*). Over the course of this century, leukemia rates have also generally increased. The incidence of leukemia grew steeply between 1900 and 1940 (*11*), and in Denmark increased 3-fold between 1943 and 1977, primarily because of increases in AML (*12*). Between 1969 and 1977, AML also increased 20% in the United States. Other studies indicate a rise in myeloid leukemias in other industrialized countries during the same period (*13*). Although overall leukemia rates have remained relatively stable over the last 20 years, the incidence of AML, which accounts for about 80 to 90% of acute leukemias in adults (*3,5*), has increased substantially among men over 40 years of age (*14*). The increased incidence of AML among older males and the fact that the highest rates of acute leukemia occur in industrial areas both suggest the importance of occupational and environmental risk factors. In addition, the incidence of certain forms of preleukemia, known as myelodysplastic syndromes (MDS), may be increasing, but this could actually reflect increased awareness on the part of physicians and extended use of diagnostic procedures in elderly patients and may not be due to changes in etiologic factors (*15*). However, the incidence of MDS in Danish children has been reported higher than generally assumed and approximates the incidence of childhood AML (*16*). MDS are life threatening, as illustrated by the recent death of the famous astrophysicist Dr. Carl Sagan.

Table 2. Subtypes and frequencies of acute leukemias.

Subtypes	Description	Frequency, %	
		Children	Adults[a]
AML			
M0	Minimal myeloid differentiation	2	?
M1	Poorly differentiated myeloblasts	13	10
M2	Myeloblastic with differentiation	28	40
M3	Promyelocytic	6	10
M4	Myeloblastic and monoblastic	19	15
M5	Monoblastic	21	10
M6	Erythroleukemic	1	5
M7	Megakaryoblastic	10	>5
ALL			
According to morphology (FAB)			
L1	Small and homogeneous	85	31
L2	Larger and heterogeneous	14	60
L3	Larger and homogeneous	1	9
According to immunophenotype			
Early pre-B cell	—	57	–
Pre-B cell	—	25	–
Transitional pre-B cell	—	1	–
B cell	—	2	–
T cell	—	15	–

[a]AML in adult data includes children and adults; however, as childhood AML accounts for a small fraction of all AML cases, these figures may represent adult percentages. Data modified from Pui (*4*), Lichtman (*5*), and Mauer (*7*).

Table 3. Established and potential risk factors of adult and childhood leukemia.

Risk factors	Leukemias	
	Adult	Childhood
Genetic factors	Family history	Concordance of infant leukemia in twins
Genetic syndromes		Down syndrome, Bloom syndrome, ataxia telangiectasia, Fanconi anemia, Familial monosomy 7, etc.
Ionizing radiation	Atomic bombing Nuclear accidents/testing Occupational exposure Radiotherapy Residential radon	*In utero* exposure to diagnostic X-rays Paternal preconception exposure
Chemical exposure	Benzene Petrochemicals Organic solvents Pesticides Chemotherapeutic drugs	Parental exposure to solvents/pesticides Maternal exposure to topoisomerase II inhibitors
Others	Viral infection (HTLV-1) Diet Smoking	Common infections (?) Diet (maternal and child) Parental smoking Previous maternal fetal loss Maternal age and alcohol consumption High birth weight

Assembled from Pui (*4*), Sandler and Ross (*10*), and Greaves (*18*).

Established Causes of Leukemia

Heredity, radiation, chemical exposures, and treatment with chemotherapeutic agents have been implicated in the development of leukemia. Viral infection by at least one known virus, human T-cell leukemia/lymphotropic virus type I (HTLV-1), is a well-understood cause of adult T-cell leukemia (*17*). The current etiology of leukemia was extensively reviewed last year by Sandler and Ross (*10*) and Greaves (*18*). The risk factors thought to be involved in leukemias are summarized in Table 3.

Genetic predisposition may play a major role in both adult and childhood leukemia (Table 3). Although the Leukemia Society of America emphasizes the fact that anyone may develop the disease, an increased risk exists among Eastern European Jews, and a decreased risk exists among Asians (*10*) (differences in diet and lifestyle may play a role, however). Individuals with a family history of leukemia or lymphoma have a 5.6-fold increased risk for AML (*10*). Parents of children with Down syndrome also have an increased risk of leukemia, and individuals with Down syndrome have a 10- to 20-fold increased risk and a greatly increased incidence of a particular subtype of leukemia, AML-M7 (*10*). This association may involve a potential leukemia gene called

AML1 at 21q22 (*19*). Another common genetic abnormality is the rearrangement of the *MLL* gene at 11q23, which is found among 80% of infants with leukemia (*10*). A familial form of monosomy 7 has also been recognized, in which two or more siblings develop myeloid leukemia before the age of 20 (*20*). This may involve a tumor suppressor gene on chromosome 7. As yet, however, no leukemia-specific suppressor genes have been identified, and these inherited genetic defects can explain the causes for only a small but significant proportion of acute leukemias (up to 5%) (*18*).

Another group of risk factors includes occupational and environmental exposure to radiation or chemicals (Table 3). The best established cause of leukemia among children is *in utero* exposure to diagnostic X-rays (*10*). Leukemia in adults is strongly associated with occupational exposure to ionizing radiation (*18*). Marie Curie and her daughter Irene both probably died of leukemia, and one of the greatest risks to astronauts in traveling to Mars or beyond may be leukemia from cosmic radiation exposure. There is little evidence, however, that nonionizing radiation such as electromagnetic fields (EMF) induces leukemia. Indeed, two recent studies have shown that EMF exposure is not a major risk factor for leukemia in children (*21*) or in adults (*22*).

Occupational exposure to chemicals, especially solvents containing benzene, has been associated with leukemia (*23*). Workers exposed to benzene with exposures greater than 200 ppm-year have an additional risk of developing AML, which is more than 20 times greater than that of the general population (*24*). The chemotherapeutic treatment of cancer induces secondary myeloid diseases, including AML and MDS. This induction is a major clinical problem and accounts for up to 10 to 20% of all AML and MDS cases diagnosed (*25*). Drugs presenting the most risk are alkylating agents, such as melphalan and busulfan, and epipodophyllotoxin topoisomerase II inhibitors. About 8% of patients treated with alkylating agents developed AML within 5 years after beginning treatment (*3*). Children with ALL treated with epipodophyllotoxins had a 5 to 12% cumulative risk of AML (*26*).

Because most people in the general population are not exposed to chemotherapeutic drugs or occupationally exposed to radiation or chemical solvents, exposure to these agents cannot explain the causes of the majority of leukemia and MDS cases diagnosed each year. We conservatively estimate that the causes of at least 20,000 (approximately 70%) of the 28,000 new leukemia cases that develop annually in the United States are unexplained. Thus, the causes of leukemia remain largely unknown. Although some success has been achieved in treating leukemias, especially in children, mortality rates have remained relatively high (approximately 75% in the United States) (*9*). Further, treatment may cause long-term damage and increased morbidity. Leukemias, therefore, place an enormous financial burden on society and cause serious psychologic trauma for many families (*27*). Identifying the causes of leukemia is therefore an important public health concern, as it could lead to the eventual prevention of this disease. Traditional epidemiologic studies have largely failed to identify the causes of leukemias in the general population. We have taken a molecular epidemiologic approach, in which traditional epidemiologic methods are combined with the latest tools of molecular biology and cytogenetics, in investigating the causes of leukemia. In recent years, our laboratory has been searching for potential biomarkers of benzene exposure that may be used to find the causes of leukemia in the general population. Benzene has served as a model environmental leukemogen in these studies.

Benzene as a Model Chemical Leukemogen

Benzene's toxic effects on the marrow were first described in 1897 (*28,29*) and the first case report of leukemia from benzene appeared in 1928 (*30*). The ability of benzene to cause AML was first fully established in the 1970s following epidemiologic studies in Italy and Turkey (*23,31–33*). There have been numerous reports of smoldering leukemias and preleukemias produced by benzene (*23*). These would likely be classified as MDS today. Recent studies in China, led by Hayes and Yin (*34,35*) and jointly sponsored by the NCI and the Chinese Academy of Preventive Medicine (CAPM), have established that benzene causes AML and MDS in humans and have also suggested that benzene exposure may be associated with non-Hodgkin's lymphoma, lymphocytic leukemia, lung cancer, and nasopharyngeal cancer.

Benzene is an important commercial product, with approximately 2 billion gal produced annually in the United States. It is used mainly as a starting material in the synthesis of numerous chemicals. The main public health issue concerning benzene in the United States and other developed countries is its use as a component of gasoline and the fact that the shift to unleaded gasoline has tended to increase its benzene content (*36–42*). In the United States, the current benzene content of gasoline is generally below 1%, but in other countries super unleaded gasoline can contain greater than 5% benzene (*43*). Another major source of public exposure to benzene is cigarette smoking. A pack-a-day smoker inhales approximately 2 mg/day, and nonsmokers who live, travel, or work with smokers are exposed to benzene through side-stream or second-hand smoke (*44*). Because benzene is also present in many foodstuffs, the background level of benzene intake for nonsmokers has been estimated at 0.5 mg/day (*45*). It is therefore difficult, if not impossible, to avoid exposure to benzene. Furthermore, benzene and solvents containing more than 1% benzene continue to be used in many countries, including China, former members of the Soviet Bloc, South America (*46–49*), and even Spain, where a case of benzene-induced aplastic anemia was recently described (*50*).

Biomarkers in the Molecular Epidemiology of Benzene-Exposed Workers

Biomarkers are indicators of molecular and cellular events in biologic systems and may allow epidemiologists to better examine relationships between environmental hazards and human health effects. Biomarkers can be classified into three categories: biomarkers of exposure, biomarkers of susceptibility, and biomarkers of early effect. Along with colleagues from the the CAPM in Beijing, the Shanghai Hygiene Anti-Epidemic Center, the NCI, 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 *a*) biomarkers of exposure to benzene, which include urinary levels of benzene metabolites, DNA adducts, protein adducts (such as albumin or hemoglobin adducts), etc.; *b*) molecular markers of susceptibility to benzene, such as inherited genetic factors or defects and polymorphisms of enzymes involved in the metabolism of benzene, including cytochrome P4502E1 (CYP2E1), myeloperoxidase (MPO), NAD(P)H:quinone oxidoreductase (NQO1), glutathione *S*-transferase (GST), etc.; and *c*) biomarkers of the early effects of benzene, including hematotoxicity (complete blood cell counts), gene mutations (glycophorin A [GPA] and *ras*, etc.), and chromosome

aberrations detected by fluorescence *in situ* hybridization (FISH), G-banding, and a micronucleus assay. An overview of the studies has been presented previously (*51*), and only those findings pertaining to susceptibility and identification of early effects will be discussed here, along with the generalizability of the findings to date.

Biomarkers of Susceptibility to Benzene Hematoxicity

As described previously, individuals with genetic defects or syndromes are highly susceptible to leukemias, though only a small proportion of leukemia cases involve such inherited susceptibility. It is possible that in a much larger percentage of cases, inherited polymorphisms in genes that encode carcinogen activation and detoxification enzymes, such as the cytochrome P450s and GST, could contribute indirectly to the leukemia risk. Multiple clinical reports suggest that people vary greatly in their susceptibility to health risks from benzene exposure. One possible reason might be interindividual variation in metabolic activation and detoxification of benzene and its primary metabolites.

Several enzymes that are involved in benzene metabolism and clearance have been identified. Benzene is metabolized by the hepatic enzyme CYP2E1 to benzene oxide, which spontaneously forms phenol. Phenol, in turn, is further metabolized by CYP2E1 to di- and trihydroxybenzenes such as hydroquinone (HQ), catechol (CAT), and 1,2,4-benzenetriol (BT) (*52*) (Figure 1). CYP2E1 therefore plays an essential role in benzene toxicity by activating it to potentially toxic metabolites (*53,54*). On the other hand, GST can detoxify benzene oxide by converting it to a less toxic or nontoxic derivative, phenylmercapturic acid (*55*). The polyhydroxy metabolites HQ, CAT, and BT are further converted in the bone marrow by MPO to benzoquinones, which are potent hematotoxic and genotoxic compounds (Figure 1). Benzoquinones can, in turn, be converted back to less toxic hydroxybenzenes by NQO1 (*53,56*) (Figure 1).

Between 5 and 20% of people in a given population may lack significant NQO1 activity (*57–59*), potentially making them susceptible to benzene toxicity. This variation is caused by a homozygous mutation ($^{609}C \rightarrow T$) at position 609 in the *NQO1* gene, which occurs among 5 to 6% of Caucasians and African Americans and as many as 18 to 20% of Chinese and other Asians (*59-61*). To test the hypothesis that individuals who were homozygous for the $NQO1^{609}$ mutation and possessed high CYP2E1 activity would be susceptible to benzene hematotoxicity, a case–control study of occupational benzene poisoning was conducted (low white blood cell count $\leq 4000/mm^3$) in Shanghai (*51,58*). CYP2E1 activity was estimated by the fractional excretion of chlorzoxazone in 50 cases of benzene poisoning and 50 controls. Subjects with both a rapid fractional excretion of chlorozoxazone and homozygous *NQO1* mutant alleles were at a 7.6-fold increased risk of benzene poisoning (*58*). We are also currently investigating the role of the *NQO1* $^{609}C \rightarrow T$ mutation in acute leukemia in general, including therapy-related leukemias. Preliminary evidence suggests that the *NQO1* polymorphism is a risk factor for some types of therapy-related leukemia.

MPO activates all the phenolic metabolites of benzene to highly toxic free radicals and quinones (*62–64*). MPO is an enzyme found primarily in neutrophils and their precursors. An inherited polymorphism in the *MPO* gene has recently been described (*65*). The polymorphism is a single base substitution (G to A) in an *Alu* repeat in the promoter region of the *MPO* gene. The presence of an A rather than a G at this site decreases expression by about two-thirds in homozygous mutant individuals (*65*). Theoretically, then, people who have mutant homozygous alleles in *MPO* should be at lower risk of benzene hematotoxicity. This hypothesis is being tested in our laboratory using a new restriction fragment length polymorphism/polymerase chain reaction (PCR) method for detecting the mutant allele (*66*). Interestingly, this new method has recently been used to show that individuals with homozygous mutant alleles in the *MPO* gene are at significantly decreased risk of lung cancer. Further, earlier studies using sequencing showed that cases of AML-M3 and AML-M4 have a decreased incidence of the mutant allele, also suggesting that homozygous mutant individuals would be resistant to these subtypes of AML (*65*). However, this analysis was based on only eight cases of AML-M3 and -M4 and requires confirmation.

The potential role of GST polymorphisms in benzene hematotoxicity is currently unclear. The GST-μ (GSTM1) and GST-θ (GSTT1) subclasses are especially effective at detoxifying epoxides, including benzene oxide that is converted to nontoxic phenylmercapturic acid (Figure 1) (*55*). Recent data, however, suggest that GSTs will not provide protection against benzoquinone metabolites of benzene because the glutathione conjugates of these metabolites are also hematotoxic (*67*). Although one study reported that the *GSTT1* null genotype (homozygous gene deletion) was associated with an increased risk of MDS (*68*), a larger, more recent study did not find such an association (*69*). Clearly, the role of GSTs in susceptibility to benzene hematotoxicity and to acute leukemia and MDS deserves further study.

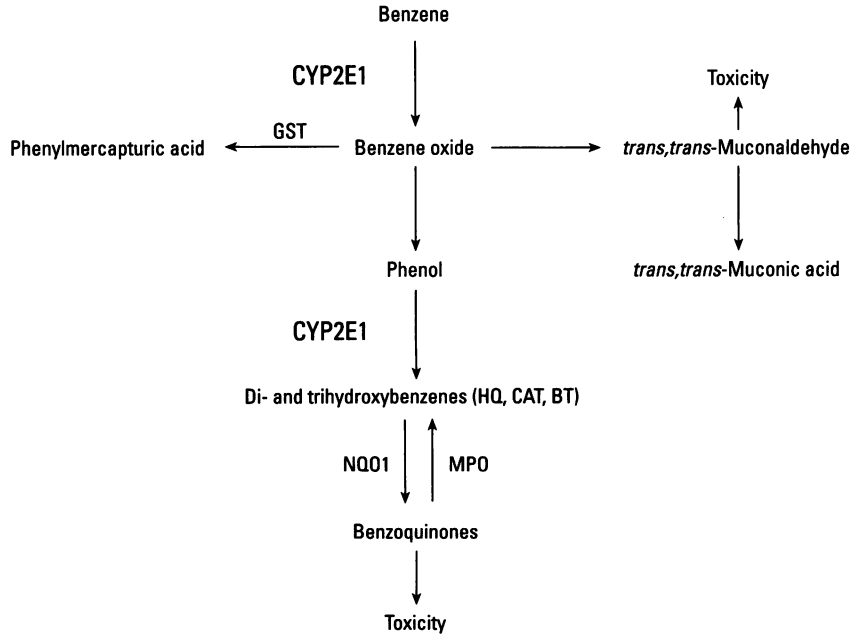

Figure 1. Pathways of benzene metabolism leading to toxicity and detoxification.

A summary of the potential role of different genetic polymorphisms and metabolizing enzyme activities in susceptibility to benzene toxicity is provided in Table 4. In theory, individuals with high activities of CYP2E1 and MPO and homozygous mutations in the *NQO1* and *GSTT1/GSTM1* genes would have the highest risk for benzene hematotoxicity.

Biomarkers of Early Effect from Benzene Exposure

Another 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 *GPA* (*70,71*). An increased *GPA* mutation frequency has been found in children with leukemia and in people exposed to radiation and leukemogenic anticancer drugs (*72*). An increased level of gene-duplicating mutations in *GPA* has also been found in benzene-exposed workers (*73*). Interestingly, this increased mutation frequency was correlated with cumulative exposure to benzene. Because cumulative exposure to benzene may correlate best with leukemia risk, the *GPA* assay appears to have potential as a biomarker of early biologic 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 (*73*). 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, including the classic early studies of Tough and co-workers (*74,75*) and Forni and colleagues (*76,77*), have demonstrated the clear association between benzene exposure and increased levels of chromosome aberrations in peripheral blood cells. More recent studies have suggested that benzene may induce aberrations at TWA concentrations below 10 ppm (*78–83*) and have selective effects on certain chromosomes (*84–87*). We are currently investigating the utility of these data in improving the risk assessment for benzene. Because chromosome aberrations in peripheral blood lymphocytes are associated with increased risk for overall cancer incidence (*88*), especially for increased mortality from hematologic malignancies (*89*), it is possible that specific chromosome aberrations may provide even better markers of future leukemia risk.

Specific Chromosome Aberrations as Biomarkers of Leukemia Risk

Specific chromosome aberrations are the hallmark of human leukemia (*90–92*). Aneuploidy, the loss or gain of specific chromosomes in AML and MDS (such as trisomy 8 and monosomy 5 and monosomy 7), is commonly observed, as are specific chromosome translocations, inversions, and deletions [e.g., t(8;21), t(9;22), inv(16), and long-arm deletion of chromosome 5] (*91*). Up to 65% of acute leukemias contain nonrandom somatically acquired chromosomal translocations or inversions (*93*). These numerical aberrations and structural rearrangements affect gene expression in ways that subvert normal cell proliferation, differentiation, and survival.

The loss of chromosomes 5 and 7 and their long-arm deletions are the two most common changes in therapy-related AML (t-AML) and MDS, especially among patients previously treated with alkylating agents (*94*). Treatment with topoisomerase II inhibitors is associated with balanced chromosome aberrations, such as t(4;11), t(6;11), and t(11;19), in t-AML (*94,95*). 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.) (*96,97*). For example, one recent study found an association between monosomy 7/long-arm deletion of chromosome 7 (del[7q]) and previous exposure to paints (odds ratio 7.5) (*97*). In addition, trisomy and monosomy of the C-group chromosomes (6-12, X) were present in the bone marrow and blood of several benzene-induced AML patients (*98–101*). Among these cases, clonal expansion of trisomy C, identified as trisomy 9 (*98*), and of trisomy D (*100*) were observed in all leukemic cells examined. Monosomy 7 was also found in 100% of the bone marrow cells of one of the benzene-induced MDS cases (*102*). Interestingly, the Philadelphia chromosome was observed by classical cytogenetics in a case of preleukemia (leukopenia) resulting from chronic exposure to benzene for 4 years without the signs of leukemia; after 4 years without exposure, the aberration disappeared (*101*).

Thus, specific chromosome aberrations have been observed in both leukemia and preleukemia patients previously exposed to benzene. However, our studies have addressed an additional question: whether benzene exposure induces these specific chromosome aberrations, which might lead to the development of leukemia in exposed but nondiseased individuals. In answering this question, we believe that measuring disease-specific chromosome aberrations in exposed workers would be more significant than measuring general nonspecific aberrations, not only because disease-specific chromosome aberrations probably have better predictive value, but because recent studies in our laboratory suggest that chemicals cause aneuploidy of specific chromosomes or produce greater damage to some chromosomes than to others (*103*). Most previous studies measured only general chromosome aberrations in benzene-exposed workers by conventional cytogenetic analysis (*78–80*). The classic assay, however, allows few cells to be examined, requires highly trained personnel, and does not readily detect specific chromosome aberrations.

Table 4. Susceptibility to benzene hematotoxicity: hypotheses on polymorphisms of enzymes involved in metabolic activation of benzene and its detoxification.

Susceptibility to benzene hematotoxicity	Activation		Detoxification	
	CYP2E1	MPO	NQO1	GSTT1/GSTM1
High	High	High	Homozygous	Homozygous
Medium	High	?	Wild-type/heterozygous	?
	Low	?	Homozygous	?
Low	Low	Low	Wild-type/heterozygous	Wild-type/heterozygous

?, uncertain. Data from Ross (*53*), Rothman et al. (*58*), and our current hypotheses.

Detection of Specific Chromosome Aberrations by FISH

Specific chromosome aberrations can now be detected by FISH (*104–106*). FISH offers several major advantages (*107*) over

conventional chromosome aberration analysis: FISH requires less-highly trained personnel; FISH is easier to perform, allowing analyses to be performed in less time; FISH analysis of metaphase cells is simpler, making it possible to analyze 10 times more metaphases; and FISH can detect very specific events identical to those found in leukemia cells. These types of events may be more strongly associated with subsequently developing leukemia than overall estimates of damage. FISH can therefore be used to detect leukemia-specific aberrations in a timely, sensitive, and cost-effective manner.

However, like conventional cytogenetic methods, metaphase analysis by FISH can only be performed on dividing cells. In peripheral blood, the cells that can most readily be stimulated to divide are the T lymphocytes; therefore, the most common technique used is cytogenetic analysis of metaphases from these cells. Peripheral T lymphocytes, though a target of the hematotoxic effects of benzene (*108,109*), are clearly not the target of genotoxic damage responsible for the development of AML and MDS. Thus, the validity of measuring AML-specific chromosome aberrations in peripheral T cells might be questioned. However, T cells might be considered a useful surrogate target because at least a portion are relatively long lived (> 1 year) and accumulate aberrations. Further, chromosome aberrations that confer selective advantages on cells of the myeloid lineage [e.g., del(7q), t(8,21)] should have no effect on T lymphocytes. Hence, detection of specific aberrations in T-cell metaphases is a measure of the number of cumulative critical hits that have occurred in the blood, and presumably the bone marrow, of control and exposed individuals on a per-cell basis. Specific chromosome aberrations in circulating T lymphocytes, which act as long-lived surrogates for stem cells in the marrow, may therefore serve as useful biomarkers of leukemia risk for benzene.

We applied FISH to determine the presence of specific chromosome aberrations in the lymphocytes of workers exposed to benzene and matched controls. Initially, we studied hyperdiploidy levels of chromosome 9 in interphase cells because trisomy 9 had been observed in benzene-poisoned patients (*98,110*) and benzene metabolites induce hyperdiploidy of this chromosome in cultured lymphocytes *in vitro* (*111,112*). High benzene exposure increased hyperdiploidy of chromosome 9 in the lymphocytes of otherwise healthy workers, with trisomy 9 being the most prevalent form (*113*). We have used interphase cytogenetics to study the hyperdiploidy of chromosomes 7 and 8. The findings were briefly reported in abstract form (*114*) and will be published elsewhere. Interphase cytogenetics cannot be used, however, to confidently detect monosomy or rare translocations because of artifacts related to probe overlap (*104*). Monosomy 5 and 7 and translocations (8;21) are among the most common aberrations observed in AML (*90,91*). We have therefore begun to use chromosome painting and region-specific fluorescent probes to examine AML-specific aberrations, including monosomy 5, monosomy 7, del(5)(q31), del(7)(q22q34), and t(8;21), in metaphase spreads prepared from the lymphocytes of workers exposed to benzene and matched controls. Increased frequencies of t(8;21) and trisomy 8 and 21 have been detected among workers exposed to benzene (*115*). Monosomy 5 and 7 and their long-arm deletions also increased in the exposed workers (*116*). Table 5 briefly summarizes the specific chromosome aberrations observed in AML and MDS detected in the benzene-exposed workers by FISH.

Detection of Specific Chromosome Aberrations by PCR-Based Technology

Specific chromosomal aberrations can also be detected by PCR and reverse transcriptase (RT)–PCR (*117–120*). These methods hold a number of advantages over FISH, including the ability to detect very rare events (1 copy/10^7 cells vs $1/10^4$ cells by FISH) and the ability to study large numbers of people easily and at low cost. These seemingly potent advantages are offset, however, by two major disadvantages. First, the high sensitivity of PCR makes it prone to false-positive results caused by sample contamination. Second, quantitation is difficult, especially for RT–PCR. The former drawback can be overcome with extremely rigorous lab procedures, but the latter is mainly restricted to a qualitative value such as number of individuals giving positive results. Because chromosomal translocations involve the formation of novel messenger RNAs and fused DNA sequences, these aberrations have been those mainly detected by PCR-based procedures capable of identifying the novel but rare sequences in millions of normal sequences. Liu et al. (*118*) demonstrated that the *BCL2* translocation [t(14;18)], commonly found in patients with non-Hodgkin's lymphoma, could be detected in the blood of healthy individuals. Biernaux et al. (*121*) observed similar results in 117 normal subjects tested by RT–PCR for the presence of *BCR-ABL* fusion in RNA from the t(9;22) (q34; q11) translocation. In both studies the translocation could be detected in up to 40% of normal healthy subjects, and its presence increased in frequency with age. PCR-based procedures therefore hold great promise for detecting specific chromosome aberrations, especially when used in combination with FISH. We are currently attempting to detect translocations t(14;18), t(9;22), t(8;21), and t(11q23) by both PCR and FISH in the peripheral blood of workers highly exposed to benzene and matched controls.

Benzene and Childhood Leukemia

Clusters of childhood leukemias have occurred around Superfund sites (*122*), and in Britain, Knox (*123*) reported that cases of childhood leukemia commonly occur closer to industrial installations. He concluded: "The common patterns of close association of clustered and nonclustered cases imply a common etiological component arising from a common environmental hazard—namely the use of fossil fuels, especially petroleum" (*123*). This work implicates benzene and petroleum products in the development of childhood leukemia, but the findings are highly controversial and were challenged in the literature (*124*). Recently, Knox and co-workers (*125*) expanded on their original findings and examined relationships between addresses at birth and death of children dying from leukemia and other cancers in Britain and the sites of potential environmental hazards. They studied all 22,458 children 0 to 15

Table 5. Specific chromosome aberrations observed in AML/MDS are detected in benzene-exposed workers.

Chromosome aberration	AML/MDS	Benzene-exposed
Aneuploidy	Trisomy 8 and 21	+7, +8, +9, +21
	Monosomy 5 and 7	–5, –7
Long-arm deletion	5q–, 7q–	5q–, 7q–
Translocation	t(8;21), t(9;22)	t(8;21)
Inversion	inv(16)	Not done

Data from Le Beau (*90*), Hagemeijer and Grosveld (*91*), Zhang et al. (*113,114,116*), and Smith et al. (*115*).

years of age who died from leukemia or cancer in England, Wales, and Scotland between 1953 and 1980. They found that childhood cancers were geographically associated with two main types of industrial atmospheric effluent, namely: petroleum-derived volatiles and kiln and furnace smoke and gases, as well as effluents from internal combustion engines. These findings support their earlier conclusion that benzene exposure is responsible for at least a portion of childhood cancers. There seems to be no positive association, however, between car ownership and childhood ALL (*126*).

The findings of Knox and co-workers are consistent with earlier reports from Holland (*127*), China (*128*), the United States (*129*), Britain (*130*), and Japan (*131*) of an association between parental exposure to solvents containing benzene and increased risk of childhood leukemia. The study in Britain (*130*) utilized face-to-face interviews for exposure assessment and found an odds ratio for parental benzene exposure as high as 5.81 (95% confidence interval [CI] 1.67–26.44). These studies imply that benzene or its metabolites cause genetic damage in female or male germ cells, which is then passed on to the offspring or causes direct genetic damage in the fetus following maternal exposure. They also imply that key changes related to the development of childhood leukemia occur before birth. This idea is strongly supported by the work of Ford, Greaves, and co-workers, which has clearly shown that genetic changes related to leukemia development occur before birth in a number of cases (*132*), including twins who developed T-cell leukemia at 9 years of age (*133*). Benzene crosses the placenta, and reproductive studies in both humans and rodents have shown that benzene exposure of either the male or the female can have harmful effects on the fetus (*134,135*). The idea that exposure of the male can lead to leukemia in the offspring is supported by the recent, quite startling finding that paternal preconception smoking was related to a significantly elevated risk of childhood cancers, particularly acute leukemia and lymphoma (*136*). The risks rose with increasing pack-years of paternal preconception smoking for ALL (p for trend = 0.01) and total cancer (p for trend = 0.006). Compared with children whose fathers had never smoked cigarettes, children whose fathers smoked more than 5 pack-years prior to their conception had adjusted odds ratios of 3.8 (95% CI = 1.3–12.3) for ALL. Clearly, more studies are needed of the relationship between parental benzene exposure and childhood leukemia, but evidence is mounting that parental genotoxic exposure is important and that key changes involved in the subsequent development of childhood leukemia can occur before birth.

Biomarkers of Childhood Leukemia Risk

We are studying a large number of cases of childhood leukemia in Northern California using molecular approaches and subclassification. FISH and PCR are being used as tools to subclassify leukemias into cytogenetic or molecular subtypes and help determine etiology, as first suggested many years ago by Kessler and Lilienfeld (*137*) and expanded upon by Sandler and Collman (*11*). Further, we aim to examine whether certain cytogenetic changes are present at birth, as is suggested by the research findings described previously. If key genetic changes occur *in utero* or are inherited from one or both parents, we may be able to detect these changes at birth using analysis of neonatal blood spots (Guthrie cards) from leukemia cases by PCR (*138*). If specific changes are detectable, it may be possible in the future to predict which children are most at risk of subsequently developing leukemia. Recently, Gale et al. (*138*) have reported that t(4;11) *MLL-AF4* gene fusion sequences can be detected in neonatal blood spots of all patients 0.5 to 2 years of age.

Conclusion

Biomarkers of susceptibility to benzene-induced hematotoxicity have been developed and more will surely be forthcoming. We and others are testing the utility of these biomarkers in predicting who is at risk for hematotoxicity and leukemia from occupational and other environmental exposures. FISH and PCR-based procedures, which measure the early effects of benzene and specific chromosome aberrations, also hold promise in predicting who is most at risk from exposure to benzene and other potential leukemogens. This endeavor deserves long-term study and is a future goal of our laboratory.

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