CHROMOSOME DAMAGE FROM BIOLOGICAL REACTIVE INTERMEDIATES

OF BENZENE AND 1,3-BUTADIENE IN LEUKEMIA

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INTRODUCTION

The causes of leukemia remain largely unknown. Only ionizing radiation, benzene and various chemotherapy drugs are established causes in humans. While benzene is an established cause of acute myeloid leukemias, its ability to cause lymphocytic leukemias and the closely related lymphomas and multiple myeloma remains highly controversial (1-4). The concentration at which it significantly increases leukemia risk is also contentious. For example, it was recently claimed in a leading journal that benzene has a dose threshold of 200 p.p.m.-years for leukemia induction (4), although there is clearly information that benzene induces leukemia and other hematological changes at much lower doses than this (3, 5) and that its effects are likely to be linear down to background levels (6). Other occupational and environmental chemicals are suspected leukemogens, but are not established as such and controversy surrounds their classification as human carcinogens. One such compound is 1,3-butadiene. This compound is widely used in the rubber industry and is a product of incomplete combustion. Its' potential classification as a human carcinogen on the basis of epidemiological studies (7, 8) showing higher rates of leukemia in exposed workers is under consideration by numerous agencies (9). To throw further light on these important questions concerning the human risk posed by benzene and butadiene, we have examined the ability of benzene, butadiene and their reactive intermediates to produce the types of chromosome damage thought to be etiologically important in leukemia and lymphoma in exposed humans and in human cells *in vitro*. This paper summarizes our recent findings and discusses their impact on the risk assessments for benzene and butadiene.

t (15 ;17)   AML
inv(16)

5q-, 7q-   AML/MDS

-5, -7, +8   AML

t (9 ;22)   CML

Myelocytes

Myeloid
Progenitor

Erythrocytes

Stem
Cell
CD34+

t (7 ;?)   T - Cell
t (14 ;?)   leukemia

inv(7)   Lymphoma

T - cells

t (14 ;18)   Lymphoma

t (12 ;21)   ALL

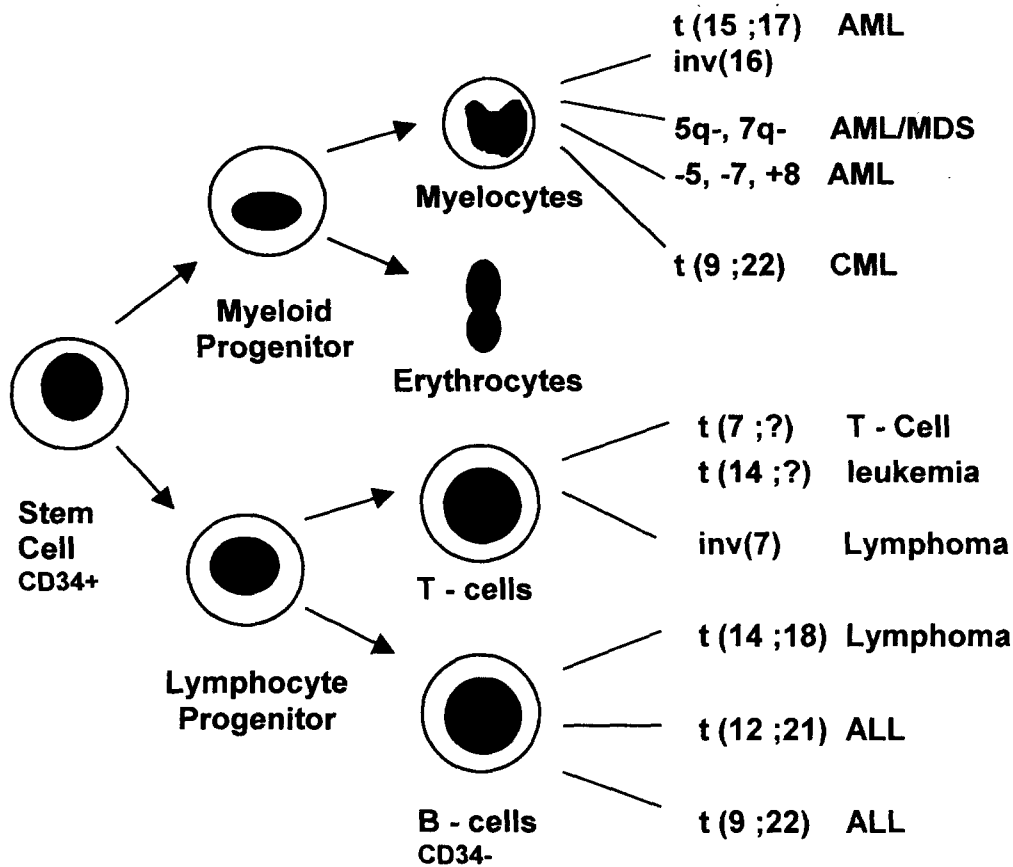Lymphocyte
Progenitor

B - cells
CD34-

t (9 ;22)   ALL

Figure 1. Clonal chromosome aberrations in the development of leukemia and lymphoma. Chromosome damage occurs in the cycling stem cells that are CD34-positive (CD34+) which progresses into leukemias of different types. AML = Acute myeloid leukemia; MDS = myelodysplastic syndromes; CML = chronic myeloid leukemia; and, ALL = acute lymphocytic leukemia

CHROMOSOME ABERRATIONS IN LEUKEMIA AND LYMPHOMA

Leukemias and lymphomas are characterized by clonal chromosomal aberrations that appear to have a central role in tumorigenesis (10, 11). In myeloid leukemia, loss of part or all of chromosomes 5 and 7 is a common early event, along with trisomy 8 and various specific translocations and inversions (Figure 1). In acute myeloid leukemia (AML), t(8;21), t(15;17) and t(11q23) are common (10, 11). Whereas in acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML), the Philadelphia chromosome t(9;22) is relatively common. In lymphocytic leukemias and non-Hodgkins lymphomas translocations and aneuploidies are also common, with the translocation t(14;18) being associated most often with follicular lymphoma (12, 13) (Figure 1). These chromosomal changes can be detected by fluorescence *in situ* hybridization (FISH) and/or the

polymerase chain reaction (PCR) (14) and may serve as biomarkers of early effect for benzene and other suspected leukemogens, including 1,3-butadiene.

SPECIFIC CHROMOSOME ABERRATIONS IN WORKERS EXPOSED TO BENZENE

We have used FISH and PCR to demonstrate elevated levels of leukemia-specific chromosome aberrations in workers exposed to high concentrations of benzene (15-18). Together with our collaborators at the Chinese Academy of Preventive Medicine in Beijing (Dr. S. Yin and G-L. Li) and at the National Cancer Institute (Drs. N. Rothman and R. Hayes) we studied a group of Chinese workers exposed to widely varying levels of benzene in Shanghai and a group of controls. Biological samples were collected from 44 healthy workers currently exposed to benzene with minimal exposure to toluene and other aromatic solvents. The same number of healthy controls without current or previous occupational exposure to benzene were enrolled from factories in the same geographic area. Controls were frequency-matched by gender and age (5 year intervals). The median benzene air level among the exposed workers was 31 ppm as an 8 hour TWA (range: 1-328 ppm). Air monitoring data were confirmed by measures of urinary benzene metabolites which showed strong, positive correlations with air benzene levels, and were substantially higher in exposed workers compared to controls (5, 19). We painted chromosomes 8 and 21 in lymphocyte metaphases from 43 workers exposed to benzene and 44 matched controls. To examine dose-response relationships the workers were divided into 2 groups at the median exposure level, a lower-exposed group (≤ 31 ppm, n = 21) and a higher-exposed group (> 31 ppm, n = 22). Benzene exposure was associated with significant increases in hyperdiploidy of chromosome 8 (1.2, 1.5, 2.4 per 100 metaphases; $P_{trend} < 0.0001$) and 21 (0.9, 1.1, 1.9; $P_{trend} < 0.0001$) (15). Translocations between chromosomes 8 and 21 were increased up to 15-fold in highly exposed workers (0.01, 0.04, 0.16; $P_{trend} < 0.0001$). In one highly exposed individual these translocations were reciprocal and were detectable by reverse-transcriptase PCR (15). These data indicate a potential role for t(8;21) in benzene-induced leukemogenesis and are consistent with the hypothesis that detection of specific chromosome aberrations may be a powerful approach to identify populations at increased risk of leukemia from benzene exposure.

We also used a novel FISH procedure to determine if specific aberrations in chromosomes 1, 5 and 7 occured at an elevated rate in metaphase spreads prepared from the lymphocytes of the same benzene-exposed Chinese workers (17). We found that benzene exposure was associated with increases in the rates of monosomy 5 and 7 but not monosomy 1 ($p < 0.001$; < 0.0001; and 0.94, respectively) and with increases in trisomy and tetrasomy frequencies of all three chromosomes. Long arm deletion of chromosomes 5 and 7 was increased in a dose-dependent fashion ($p = 0.014$ and < 0.0001) up to 3.5-fold in the exposed workers. These results demonstrate that leukemia-specific changes in chromosomes 5 and 7 can be detected by FISH in the peripheral blood of otherwise healthy benzene-exposed workers. Studies are being planned to apply these methods in workers exposed to lower levels of benzene. In addition, we plan to explore the impact of inter-individual variation in genes that activate and detoxify benzene and its metabolites on these outcomes.

Toxicoepidemiology can be defined as the study of the adverse effects of chemicals using epidemiological methods. One of the goals of the above toxicoepidemiological studies is to improve the risk assessment for benzene by providing important scientific information. It is unlikely that classical epidemiological studies of cohorts of workers historically exposed to benzene will shed light on the dose response curve at exposure levels below 10 p.p.m. in air, because a very large number of workers would have to be studied (> 100,000) and the assessment of historical exposures is an inexact science in which only ranges can be estimated. Biomarker studies hold the advantage of requiring much fewer study participants and there is no need to wait for the disease to develop as a surrogate biomarker is used. The surrogate marker is assumed to be a predictor of future risk. Fortunately, for cancer and hematological malignancies especially, chromosome aberrations have been shown to be predictive of future risk (20). Our goal in using biomarkers to study a large number of workers exposed to around 1 p.p.m. benzene is therefore to establish the dose response curve for specific chromosome aberration induction in the 0.5 to 10 p.p.m. range. By inference, we suggest that this will predict the likely shape of the dose response curve for benzene induced hematological malignancies in this same low dose range.

Biomarkers can also shed light on other questions of importance to benzene risk assessment. One important question is: "Are there susceptible individuals?" Our studies in China have shown that workers with high cytochrome P4502E1 and no NQO1 activity were at a 7.8 fold higher risk of benzene hematotoxicity, a condition that predisposes to leukemia (21). This indicates that at least an 8-fold safety factor should be applied in benzene risk assessment to account for susceptible individuals. Further, it is likely that other factors will be discovered that explain inter-individual differences in susceptibility and may lead to the need to increase the size of the safety factor (14).

Another important question is: "What is the toxic metabolite(s) of benzene?" This information is needed for physiologically-based pharmacokinetic models that are commonly used in risk assessment (22). However, it is unlikely at present that biomarkers can throw much light on this issue. The exact metabolite(s) responsible for benzene's effects are unknown, but it has been suggested that a combination of at least two metabolites is involved (23). It is also possible that one metabolite may be responsible for one toxic effect of benzene and a second responsible for another toxic effect. The problem is that there is no unique pathway that can be identified as being primarily responsible for the toxic effects. There is, however, considerable evidence for the involvement of the phenolic metabolites (23). For example, we have shown that several of these metabolites, most notably hydroquinone, can produce many of the chromosomal changes found in leukemia *in vitro* in human lymphocytes (24) and most recently in human CD34-positive early progenitor / stem cells (25). Further, we demonstrated that exposure to hydroquinone produced monosomy and trisomy of chromosomes 7 and 8 to a greater extent and at lower concentrations in CD34+ than in CD34- cells (25). Particularly striking effects of hydroquinone were observed in CD34+ cells on monosomy 7 and trisomy 8, two common clonal aberrations found in myeloid leukemias, suggesting that these aneusomies produced by hydroquinone in CD34+ cells play a role in benzene-induced leukemogenesis. The reason for the greater sensitivity of CD34+ cells to hydroquinone genotoxicity is unclear at this time, but may be related to a lack of certain protective enzymes, such as NAD(P)H:quinone oxidoreductase (26, 27).

It is also possible that certain forms of DNA repair are lacking or are at lower levels in these immature progenitor cells. It has been reported, for example, that CD34+ cells have lower levels of alkylguanine DNA alkyltransferase activity (28). On the other hand, it has also been shown that CD34+ cells have higher levels of nucleotide excision repair (29). Thus, the role of DNA repair in hydroquinone sensitivity, and the DNA repair status of CD34+ cells requires further investigation.

Another issue in benzene risk assessment is which disease endpoints to use: Just AML or AML plus myelodysplastic syndromes or all leukemias or leukemias plus lymphomas (22). There is currently no consensus on this issue and uncertainty as to whether or not benzene causes non-Hodgkin's lymphoma. We have therefore examined the ability of the benzene metabolite, hydroquinone, to induce the t(14;18) translocation in human cord blood lymphocytes which possess active V(D)J recombinase. The t(14;18) translocation is frequently found in follicular lymphomas and is thought to be induced by the illegitimate action of V(D)J recombinase. Isolated lymphocyte cultures were exposed to hydroquinone for 48 h and the frequency of t(14;18) translocations determined using a highly sensitive quantitative real-time exonuclease based PCR assay. A significant increase in the frequency of t(14;18) above background levels was generated by treatment with hydroquinone. Sequencing of the PCR products revealed these to be identical or similar to breakpoint sequences previously reported for t(14:18) in cell lines and clinical samples. These findings suggest that hydroquinone has the ability to induce of t(14;18) translocations in human cells with active V(D)J recombinase and provide a mechanistic basis for the induction of non-Hodgkin's lymphoma by benzene exposure. An important question now for risk assessors is how to use this mechanistic information from *in vitro* toxicological studies.

TOXICOEPIDEMIOLOGICAL STUDIES TO IMPROVE THE RISK ASSESSMENT FOR BUTADIENE

We have also used FISH to study workers exposed to the potential leukemogen 1,3-butadiene and lymphocytes exposed *in vitro* to its metabolites. The *in vitro* studies showed that the epoxy metabolites of butadiene caused selective aneuploidy of certain chromosomes (30), with 1,2,3,4-diepoxybutane being a highly effective inducer of both aneuploidy and structural chromosome damage (30, 31). Together with Richard Hayes of the National Cancer Institute, James Swenberg's group at the University of North Carolina and Chinese investigators, including Drs. Yin and Li, we have examined if aneuploidy similar to that observed *in vitro* is also found in the blood cells of workers exposed to butadiene. We studied a group of workers at a polybutadiene rubber production facility in Yanshan, China (32). In total, 41 butadiene-polymer production workers and 38 nonexposed controls, matched for age, sex and smoking status were studied. Among the butadiene-exposed workers, the median air exposure was 2 ppm (6-hour TWA), due largely to intermittent high-level exposures. Compared to unexposed subjects, butadiene-exposed workers had greater levels of hemoglobin N-(2,3,4-trihydroxybutyl)valine (THBVal) adducts (p <.0001), and adduct levels tended to correlate, among butadiene-exposed workers, with air measures (p = .03) (32). Butadiene-exposed workers did not differ, however, from unexposed workers with respect to the frequency of aneuploidy of any of the four chromosomes 1, 7 , 8 and 12 as measured by FISH (32). Also butadiene exposure and greater THBVal levels were not associated with increases in other measures of genotoxic damage namely sister chromatid

Overall, this investigation in China demonstrated that exposure to butadiene, by a variety of short-term and long-term measures, did not show specific genotoxic effects related to that exposure.

Others studies have shown genotoxic effects in workers exposed to butadiene, but the results have not been consistent. Among U.S. butadiene-styrene workers, dicentrics were significantly correlated with urinary butadiene metabolites and there was evidence of deficiencies in DNA repair by the CAT-host cell reactivation assay (34). Increased frequency of mutations in *hprt* were also observed in U.S. butadiene workers (35) but excesses were not found in our study (32, 33, 35) or in studies in the Czech Republic (36). Increased frequency of chromosomal aberrations and sister chromatid exchange were reported in the Czech Republic (36, 37), but an earlier investigation of these subjects and others showed no excesses (38, 39). Taken together these results cast doubt on the genotoxic potential of butadiene at low levels of occupational exposure. Our negative results are relevant, however, only for exposures in the butadiene exposure range studied. Also, the relatively small size of the study sample may have limited our ability to detect modest effects. It is therefore feasible that higher levels of exposure to butadiene may induce genotoxic effects, including aneuploidy, in humans. However, it is also possible that humans are far less sensitive to the genotoxic effects of butadiene than other species, as suggested by van Sittert et al. (40), because they have high liver epoxide hydrolase activity and that our negative results reflect this. The high activity of epoxide hydrolase in human liver affects butadiene metabolism such that proportionally very low levels of the highly genotoxic diepoxybutane are formed from the monoepoxy metabolite because it is rapidly detoxified to a non-genotoxic diol by the epoxide hydrolase. Further, any diepoxybutane that is formed is metabolized by the epoxide hydrolase to 1,2-dihydroxy-3,4-epoxybutane, which is much less genotoxic that diepoxybutane. The genotoxic and carcinogenic potential of butadiene in man therefore requires further investigation.

ACKNOWLEDGMENTS

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