RESEARCH REPORT

HEALTH EFFECTS INSTITUTE

Number 103
June 2001

Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans

David A Eastmond, Maik Schuler, Chris Frantz, Hongwei Chen, Robert Parks, Ling Wang, and Leslie Hasegawa

Includes a Commentary by the Institute's Health Review Committee

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HEI STATEMENT
Synopsis of Research Report 103

The Nature of Chromosomal Alterations and How They Are Induced by Benzene in Mice and Humans

INTRODUCTION

Exposure to high levels of benzene is associated with the development of leukemia and other blood disorders, but the effects of exposure to low levels of benzene are not well understood. In the 1990s, the Health Effects Institute initiated its Air Toxics Research Program to address uncertainties about the health effects that may result from exposure to ambient levels of benzene and other air toxics derived from mobile sources. One of the goals of this program was to develop and validate biomarkers of benzene exposure.

Benzene can induce changes in the structure and function of chromosomes, although the relevance of these findings to the development of clinical conditions has not been fully established. HEI funded Dr David Eastmond to investigate two related approaches to determining whether such chromosomal changes could be used as biomarkers of benzene exposure in mice and humans. HEI also thought that Eastmond's study would provide useful data to compare benzene's effects in two species.

APPROACH

The first part of the study involved detecting chromosomal alterations in cells using a modification of a molecular cytogenetic technique known as fluorescence in situ hybridization (FISH). Eastmond used two different fluorescently labeled DNA sequences ("tandem labeled probes") that would bind to unique regions of particular chromosomes. This approach, if successful, may be better than other cytogenetic methods for estimating benzene's effects because it is potentially highly sensitive and may be useful in large population studies. It could also provide information about how different chromosomal alterations arise. Eastmond and colleagues evaluated the frequency of such chromosomal aberrations in the erythrocytes (red blood cells) from the bone marrow of mice exposed to various doses of benzene (50 to 450 mg/kg of body weight per day) and for different exposure durations (2, 6, or 12 weeks). The investigators also tested aberrations in chromosomes 1 and 9 of peripheral blood cells from two groups of humans occupationally exposed to benzene who were matched with control subjects. One exposed population comprised 44 Chinese workers who were either currently being exposed to median levels of 31 parts per million (ppm) benzene, or had formerly been exposed to such high levels that they had become "benzene poisoned." The other exposed population was made up of 17 Estonian workers; 12 subjects were in benzene production (exposed to about 1.3 ppm) and 5 were operating a coke oven (exposed to about 0.3 ppm benzene).

The second part of Eastmond's proposal was to determine whether benzene or its metabolites affect DNA indirectly, acting through the nuclear enzyme topoisomerase II. This enzyme plays a key role in maintaining the chromosomal structure, so inhibiting topoisomerase II function might lead to chromosomal damage or to the development of aberrations. The investigators tested a number of benzene metabolites in vitro to assess their inhibitory effects on the purified human enzyme and on the enzyme's activity in a human cell line. They also tested whether administering benzene orally to mice would inhibit the enzyme's activity in vivo. This part of the study was expected to provide novel information about what mechanisms may be relevant to the carcinogenic effects of benzene, which are not well understood.

RESULTS AND INTERPRETATION

Eastmond and colleagues addressed several important goals in their study. Using tandem labeled fluorescent probes they demonstrated that they could detect some types of benzene-induced chromosomal

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr David Eastmond of the University of California, Riverside. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

alterations in mice and humans. Controlled exposure studies in mice suggested that benzene-induced increases in chromosomal alterations in bone marrow erythrocytes are dependent on both dose and duration of exposure. By contrast, the results of human biomonitoring were not as clearcut: Chromosomal alterations in the highly exposed population of Chinese workers did not differ from control levels, but the smaller group of Estonian workers who were exposed to lower levels did show chromosomal changes related to benzene exposure. A number of reasons may explain why the investigators found higher numbers of aberrations in the chromosomes of Estonian workers than in Chinese workers. For example, an agent or agents distinct from benzene in the Estonian work environment (such as polycyclic aromatic hydrocarbons) may be a factor; differences in lifestyle (such as diet or medications) may be influential; or an unusual dose-response curve for benzene, in which lower doses would induce higher numbers of aberrations, is an option. An additional explanation is that these two groups of workers could express different types of enzymes that may metabolize benzene along distinct pathways to harmful or less harmful metabolites. The binding of the fluorescent DNA probes to cells is also likely to be critically influenced by the way in which the slides of cell samples are prepared. Because slides for the two studies were prepared in different countries, it is quite probable that differences in preparation conditions might also have affected the results. Thus, although the results obtained by Eastmond and his colleagues indicate the feasibility of the approach tested, they also underline important limitations in the use of the tandem labeled FISH assay in large human studies.

These investigators were the first to show that benzene administration to mice in vivo, and some benzene metabolites or potential metabolites in vitro, can inhibit the nuclear enzyme topoisomerase II. These findings suggest a potential mechanism by which benzene may induce genotoxic and carcinogenic effects. Because the results of the in vitro assay of topoisomerase II activity were not linear in the dilution range tested, however, the assay cannot be used at present as an indicator of early benzene effects.

The investigators also were able to conduct initial tests of new biomarkers of benzene exposure and effects in humans. Additional studies will help to determine whether using FISH with tandem probes or measuring topoisomerase II activity will be useful biomarkers for assessing ambient or occupational exposures to benzene.

Library of Congress Catalog Number for the HEI Report Series: WA 754 R432.
The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report 21, December 1988; and effective with Report 92, the paper is recycled from 100% postconsumer waste, with Reports 25, 26, 32, 51, 65 Parts IV, VIII, and IX, and 91 excepted. These excepted Reports are printed on acid-free coated paper.

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Research Report 103

Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans

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HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

INVESTIGATORS' REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

Continued

COMMENTARY Health Review Committee

The Commentary about the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss remaining uncertainties and implications of the findings for public health.

Continued

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Publishing History: This document was posted as a preprint on *www.healtheffects.org* and then finalized for print.

Citation for whole report:

Eastmond DA, Schuler M, Frantz C, Chen H, Parks R, Wang L, Hasegawa L. 2001. Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans. Research Report 103. Health Effects Institute, Cambridge MA.

When specifying a section of this report, cite it as a chapter of this document.

INVESTIGATORS' REPORT

Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans

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ABSTRACT

Elevated frequencies of chromosomal aberrations have been observed in the lymphocytes of benzene-exposed workers. Similar changes occurring in the bone marrow may play an important role in the development of leukemia. The objective of this research has been to characterize chromosomal alterations induced by benzene in mice and humans and to investigate the potential role of inhibition of topoisomerase II in the myelotoxic effects of benzene. The research is presented in three sections corresponding to the specific aims of the project: genotoxicity studies in the mouse, topoisomerase II studies, and initial studies using a new fluorescence in situ hybridization (FISH)* approach to detect chromosome alterations in benzene-exposed workers.

The results of the mouse experiments indicate that both chromosome breakage and aneuploidy are induced in the bone marrow of $B6C3F_1$ mice following benzene administration. Chromosome breakage is the predominant effect, and this occurs primarily in the mouse euchromatin. Significant breakage within the mouse heterochromatin was also observed, as was aneuploidy. Breakage in the mouse bone marrow erythrocytes increased as a function of both dose and duration of benzene administration. The aneuploidy resulting from benzene exposure in mice was a relatively infrequent event, with increases of both chromosome loss and hyperdiploidy being observed.

In the topoisomerase studies, benzene or its metabolites were shown to inhibit topoisomerase II enzyme activity in an isolated enzyme system, in a human bone marrow-derived leukemia cell line, and in vivo in the bone marrow of treated mice. The decreased activity was probably due to the rapid degradation of the topoisomerase II protein in the treated cells.

In the human biomonitoring studies, the feasibility of using FISH with tandem DNA probes to detect chromosome alterations in interphase granulocytes and lymphocytes of benzene-exposed workers was demonstrated. The results from the two worker studies were somewhat inconsistent, however. In the study of Estonian workers, characterized by lower exposures and a smaller sample size, the benzene-exposed workers exhibited elevated frequencies of breakage in the 1q12 region as compared with those seen in controls. A suggestive trend toward increased hyperdiploidy was also seen, although the frequencies in the exposed workers were low and within the range of our laboratory's historical control frequencies. In the larger study of more highly exposed Chinese workers, no increase in breakage affecting the 1q12 region was seen among the exposed workers. A trend toward increased hyperdiploidy of chromosome 1 was seen in the exposed workers when the concentration of urinary benzene metabolites was used in conjunction with the frequency of hyperdiploidy observed in the lymphocytes of the individual workers.

The results of these studies indicate that benzene exposure is characterized by chromosome breakage, primarily within the euchromatin, and modest increases in aneuploidy. These findings also provide the first direct evidence that benzene is capable of inhibiting the enzymatic activity of topoisomerase II in vivo, providing additional support for the hypothesis that inhibition of topoisomerase II contributes to benzene-induced toxicity and leukemogenesis.

* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report 103, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr David A Eastmond, Environmental Toxicology Graduate Program, 5429 Boyce Hall, University of California, Riverside CA 92521.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R828112 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

INTRODUCTION

Benzene is a widely used industrial chemical and a ubiquitous environmental pollutant due to its presence in gasoline, tobacco smoke, and various consumer products (International Agency for Research on Cancer [IARC] 1982; International Programme on Chemical Safety [IPCS] 1993). Chronic exposure to high concentrations of this agent is associated with pancytopenia, aplastic anemia, and leukemia in humans (Agency for Toxic Substances and Disease Registry [ATSDR] 1992; IARC 1982; IPCS 1993). Prolonged exposure of laboratory animals to benzene results in myelotoxicity as well as the formation of tumors in multiple tissues (Huff et al 1989; IARC 1982; IPCS 1993).

In spite of extensive research, identification of the mechanisms by which benzene exerts its toxic and carcinogenic effects has remained elusive. Studies in animals have indicated that benzene itself is unlikely to be the actual toxicant but rather requires metabolism to exert its hematopoietic effects (Andrews et al 1977; Gad-El Karim et al 1986; Sammett et al 1979; Sawahata et al 1985; Valentine et al 1996). Early studies by Sammett and associates also demonstrated the importance of liver metabolism by showing that partial hepatectomy resulted in higher levels of benzene in the bone marrow, yet protected against toxicity (Sammett et al 1979). In addition, Irons and coinvestigators showed that very little metabolism of benzene occurred in the bone marrow (Irons et al 1980; Sawahata et al 1985). The requirement for metabolism in the liver with toxicity occurring in the bone marrow suggests that a relatively stable metabolite formed in the liver is transported to the bone marrow and exerts its toxic effects.

Additional studies have shown that bioactivation in the liver occurs primarily through oxidation by the cytochrome P450 2E1 monooxygenase system leading to the formation of phase I metabolites including phenol, hydroquinone, catechol, benzene dihydrodiol, 1,2,4-trihydroxybenzene, and *trans,trans*-muconic acid (Figure 1) (Guengerich et al 1991; Schlosser et al 1993; Seaton et al 1994; Snyder et al 1981). The involvement of P450 2E1 in the bioactivation of benzene has been convincingly demonstrated recently by Valentine and coworkers who showed that transgenic mice in which the cytochrome P450 2E1 gene (*Cyp2e1*) had been knocked out ($Cyp2e1^{-/-}$) did not exhibit myelotoxic or genotoxic effects following exposure to benzene, whereas strong cytotoxic and genotoxic effects were seen in wild type mice (Valentine et al 1996). The specific metabolite or metabolites involved have yet to be identified, however. Administration of benzene's primary metabolites to rodents has failed to produce the myelotoxicity characteristic of benzene (Eastmond et al 1987). This inability of the known benzene metabolites to exhibit extensive myelotoxicity has led investigators to investigate the role of reactive intermediates formed during ring opening such as *trans,trans*-muconaldehyde, a reactive dialdehyde that rearranges to form *t,t*-muconic acid. (Goldstein et al 1982b; Witz et al 1985). Other investigators have investigated the ability of benzene's phase II metabolites to produce myelotoxic effects. Recent studies by Monks and coworkers showed that glutathione conjugates derived from 1,4-benzoquinone, 2,3,5-tris(glutathione-*S*-yl)hydroquinone, and 2,6-bis(glutathione-*S*-yl)hydroquinone were toxic to erythroid bone marrow cells when administered to Sprague-Dawley rats (Bratton et al 1997).

Other investigators have proposed that the myelotoxic effects of benzene result from the interactive effects of various metabolites. Studies by Eastmond, Smith, and Irons demonstrated that the coadministration of phenol and hydroquinone to mice resulted in potent myelotoxic effects (Eastmond et al 1987). Subsequent studies have shown that this combination, as well as other combinations of metabolites, also exhibit significant myelotoxic and genotoxic effects (Barale et al 1990; Chen and Eastmond 1995a; Dimitriadis et al 1988; Guy et al 1990; Hu et al 1990; Kolachana et al 1993; Marrazzini et al 1994; Subrahmanyam et al 1990). Recent studies on the genotoxicity of the phenol-hydroquinone combination conducted by Chen and Eastmond have indicated that the interactive effects may not simply be due to higher concentrations of hydroquinone reaching the bone marrow but may involve an inhibition of enzymes involved in DNA replication and repair, such as the topoisomerase enzymes, by phenol, hydroquinone, or their metabolites (Chen and Eastmond 1995a,b).

A secondary bioactivation of benzene's phenolic metabolites has been proposed to occur in the bone marrow and may be responsible for the ultimate formation of the reactive myelotoxic species (Eastmond et al 1987; Sawahata et al 1985). Human and mouse bone marrow contains appreciable levels of myeloperoxidase, eosinophil peroxidase, and prostaglandin H synthase, oxidative enzymes that have been shown to be capable of converting benzene's phenolic metabolites to reactive quinone metabolites (Figure 1) (Eastmond et al 1987; Ross 1996; Schattenberg et al 1994). In addition, under certain circumstances cytochrome P450 monoxygenases as well as iron-containing molecules such as heme and hemoglobin can function as peroxidases (Anari et al 1996; Berman and Adams 1997; Segura-Aguilar 1996; Tseng and Latham 1984). During peroxidase-mediated metabolism, hydroquinone and catechol can be converted to 1,4- and 1,2-benzoquinones, respectively. Phenol can be oxidized by peroxidase enzymes to

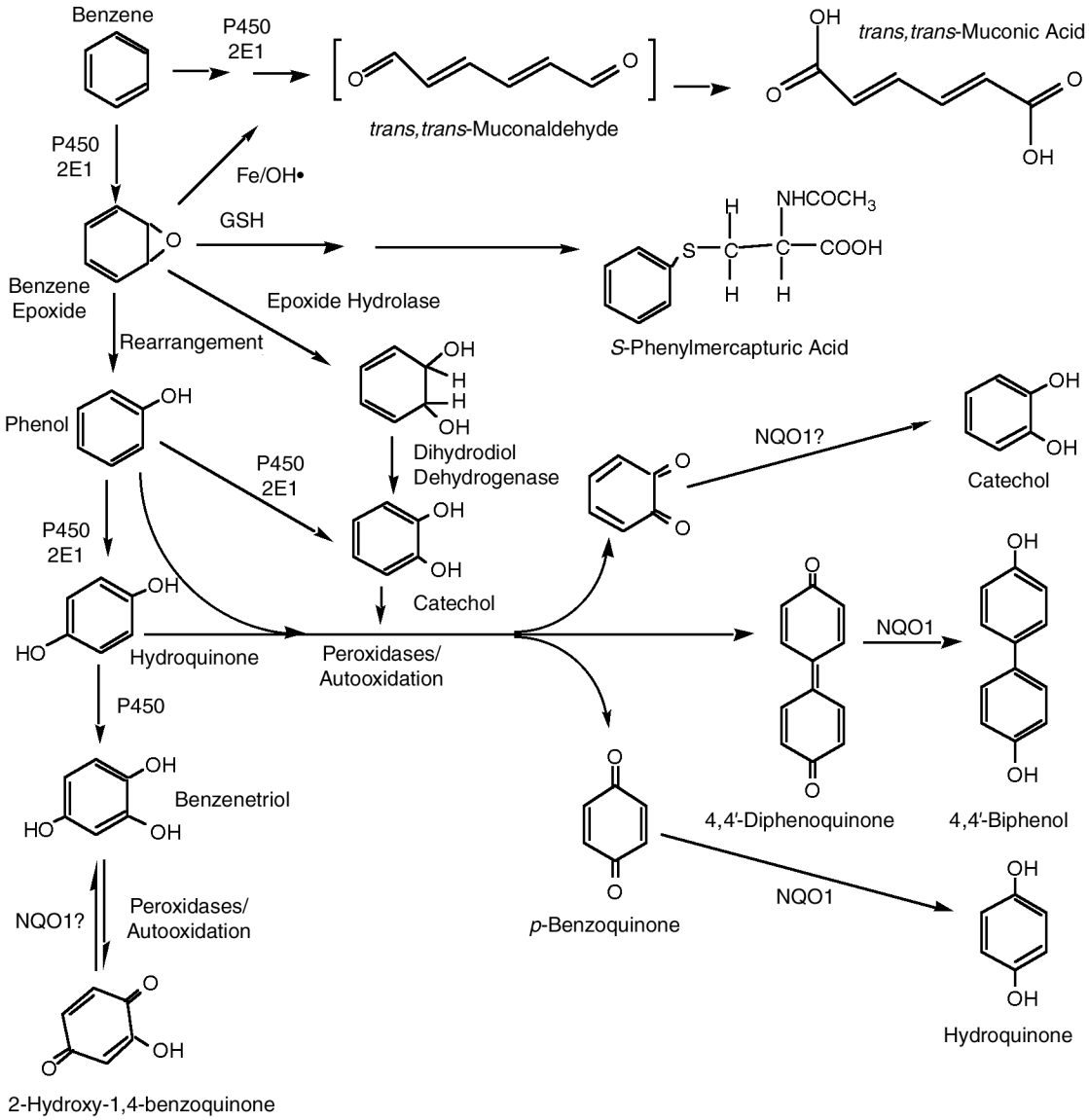

Figure 1. Metabolic pathways of benzene (adapted from Ross 1996).

form 2,2′-biphenol, 4,4′-biphenol, and diphenoquinone, as well as other oxidation products (Eastmond et al 1986; Subrahmanyam and O'Brien 1985a). These quinones are reactive electrophiles capable of binding covalently to cellular macromolecules. Evidence for the involvement of peroxidases and quinone metabolites in the myelotoxic and carcinogenic effects of benzene has been provided by two recent studies (Low et al 1995; Rothman et al 1997). In the first study, Low and associates have reported a relation between the tissue levels of a bioactivating peroxidase and the location of tumors in benzene-exposed rats. In the second, Rothman and colleagues showed that workers possessing an inactivating point mutation in the NAD(P)H: quinone oxidoreductase 1 (*NQO1*) gene, which codes for an enzyme that catalyzes a two-electron reduction of quinones to hydroquinones, had an increased risk of myelotoxicity from benzene exposure.

Because of its well-known leukemogenic properties, benzene has been the object of a large number of investigations on genotoxicity (Dean 1978, 1985; Snyder and Kalf 1994). In vitro mutagenicity testing has generally indicated that benzene and its major metabolites are weakly mutagenic or nonmutagenic in most standard gene mutation assays (IPCS 1993; Waters et al 1988). The interpretation of these studies is complicated because of the complexity of benzene metabolism and the fact that most studies have

not employed proper metabolic activation. In vivo mutagenicity studies, however, have not detected dose-related increases in mutation frequency in reporter genes in the bone marrow of treated mice (Provost et al 1996). Increases in mutation frequency have been measured in reporter genes of cells isolated from the spleens of transgenic mice (Mullin et al 1995; Provost et al 1996; Stratagene 1994) and in the endogenous *Hprt* gene of native CD-1 mice (Ward et al 1992). Higher frequencies were seen in the *Hprt* study and may reflect the increased ability of the *Hprt* assay to detect deletions as well as the intragenic or point mutations that are commonly detected in the *lacI* transgenic system.

Other in vitro genotoxicity studies that have focused on chromosome-level alterations have reported that benzene and its metabolites are capable of inducing chromosome breakage and may interfere with chromosome segregation (IARC 1982; Waters et al 1988; Yager et al 1990). Significant increases in structural chromosomal aberrations, micronuclei, and sister chromatid exchanges (SCEs) have also been detected in the bone marrow and spleens of benzene-treated animals (Chen et al 1994a; IPCS 1993; Tice et al 1980, 1981). In addition, significant increases in micronuclei originating from chromosome loss were seen in a recent single-dose study of benzene-exposed mice (Chen et al 1994a).

Similar chromosomal alterations have been observed in the peripheral blood lymphocytes of individuals occupationally exposed to benzene. Studies of benzene-exposed workers have consistently shown an association between benzene exposure and elevated frequencies of structural chromosome aberrations (Aksoy 1988; Sarto et al 1984). Moreover, increased frequencies of numerical aberrations have occasionally been reported to occur in benzene-exposed workers (Aksoy 1988; Eastmond 1993). In contrast to most studies in which structural aberrations have been observed in workers with current benzene exposure, however, the studies in which aneuploidy has been detected have generally been performed on individuals who exhibited previous bone marrow toxicity, and the studies were initiated some time after benzene exposure had ceased (Ding et al 1983; Forni et al 1971; Liniecki et al 1971; Pollini and Biscaldi 1976; Pollini et al 1969). This suggests that the observed numerical aberrations may be an effect secondary to chronic myelotoxicity or aplastic anemia rather than a direct consequence of benzene exposure.

The importance of chromosomal mechanisms in benzene genotoxicity has been supported by a recent study in which the glycophorin A (*GPA*) mutation assay was used to detect mutations in benzene-exposed Chinese workers (Rothman et al 1995). The observed mutations were exclusively of a type that originated from loss of one allele combined with duplication of the other allele. This pattern of alteration is likely to be the result of recombination or nondisjunction combined with chromosome loss. These mutations, originating in the bone marrow of exposed humans, suggest that benzene induces chromosome-level mutations rather than producing inactivating mutations within the *GPA* locus. These findings are consistent with previous studies on the genetic toxicology of benzene and its metabolites as well as human biomonitoring studies, and they provide further evidence for the hypothesis that chromosome-level genetic alterations contribute to benzene-induced leukemia.

The objective of this research was to utilize recently developed molecular cytogenetic techniques to characterize the nature and persistence of chromosomal alterations induced by benzene in mice and humans and to determine the role of topoisomerase inhibition in the formation of the observed chromosomal changes. The specific aims and a brief description of each is provided below. In the following sections, each specific aim is addressed separately with a brief introduction, and methods, results, and discussion sections.

Aim 1. Characterize the origin of chromosomal alterations occurring in B6C3F_1 mice following short-term and longer-term benzene exposure. Exposure of B6C3F_1 mice to benzene results in the formation of micronucleated erythrocytes and tumors in multiple organs including the hematopoietic system (Dean 1985; Huff et al 1989). For this aim, the nature of chromosomal alterations occurring in the erythrocytes and nucleated bone marrow cells of mice following benzene exposure was determined using newly developed FISH techniques. Studies were conducted following administration of benzene to B6C3F_1 mice for 2, 6, or 12 weeks. In addition to determining micronucleus frequencies, the persistence of hyperdiploidy, chromosome loss, breakage within the euchromatic region, and breakage within the heterochromatic region of mouse chromosomes was determined in these short- and longer-term studies.

Aim 2. Determine the role of topoisomerase II inhibition in the formation of chromosomal alterations induced by benzene. Inhibition of topoisomerase II is a mechanism by which a number of chemotherapeutic drugs exert their toxic effects. These agents are highly effective at inducing chromosomal breakage and polyploidy and exhibit other characteristics similar to those previously seen in genotoxicity studies of benzene. For this aim, the ability of benzene and its phenolic metabolites to inhibit topoisomerase II was investigated using a variety of approaches in vitro and in mice in vivo.

Aim 3. Characterize chromosomal alterations occurring in worker populations with current and previous exposure to varying levels of benzene. This section describes a series of initial studies to determine the feasibility of using a new multicolor FISH technique to detect hyperdiploidy and chromosomal breakage/exchanges occurring in interphase cells of workers exposed to benzene. In the first study, FISH with tandem DNA probes was used to assess chromosome alterations affecting the 1cen-1q12 and 9cen-9q12 regions in the lymphocytes and granulocytes of a small group of individuals working at a refinery in Estonia. In the second study, the tandem FISH technique was used to detect chromosome alterations affecting the 1cen-1q12 region in cultured lymphocytes obtained from two groups of benzene-exposed workers, one group of individuals currently exposed to high levels and a second group of individuals who previously had experienced benzene myelotoxicity and who, for the most part, had been removed from further exposure.

ORIGIN OF CHROMOSOMAL ALTERATIONS IN B6C3F$_1$ MICE FOLLOWING SHORT-TERM AND LONGER-TERM BENZENE EXPOSURE

Elevated frequencies of chromosomal aberrations have been observed in the peripheral blood lymphocytes of humans occupationally exposed to benzene as well as other carcinogenic agents (Aksoy 1988; Sorsa et al 1992). Similar types of alterations are commonly seen in the tumor cells of cancer patients, and recent molecular and cytogenetic evidence indicates that the induction of these chromosomal changes may play an important role in carcinogenesis (Solomon et al 1991). Chromosomal aberrations are generally divided into two types: (1) structural aberrations, which include changes in chromosome structure such as chromosome deletions, translocations, and inversions; and (2) numerical aberrations, which include changes in chromosome number such as chromosome loss (hypodiploidy), chromosome gain (hyperdiploidy), and polyploidy. Although it is widely recognized that the administration of benzene to laboratory animals results in increased levels of structural chromosomal aberrations in the bone marrow (Dean 1978, 1985; IPCS 1993), much less is known about the ability of benzene to induce aneuploidy in this organ. In addition, almost all of the cytogenetic studies to date have been conducted following acute exposures to benzene, so that almost nothing is known about genetic changes occurring in the bone marrow of animals with prolonged benzene exposure.

For many years, cytogenetic analyses of metaphase cells have been relied upon to detect structural and numerical aberrations in humans and animals following treatment with genotoxic agents (Carrano and Natarajan 1988; Sorsa et al 1992). Although valuable, these techniques are labor intensive, require highly skilled personnel, and are prone to technical artifacts such as chromosome loss during metaphase preparation or inadequate spreading of metaphase chromosomes. Furthermore, these techniques are limited to actively dividing cells such as lymphocytes and cannot be performed on terminally differentiated cells such as polymorphonuclear leukocytes, the cell type primarily affected in benzene-induced leukemia (Aksoy 1988). Fluorescence in situ hybridization with DNA probes is a relatively new molecular cytogenetic technique that allows cytogenetic information to be obtained from interphase as well as metaphase cells. For a review of the use of FISH in environmental mutagenesis, see Eastmond and Rupa (1995). Over the past 30 years, DNA sequences (probes) that hybridize to blocks of repetitive sequences have been identified. In situ hybridization with these probes results in brightly fluorescent spots at the position of the target DNA sequences, which can be easily detected on metaphase chromosomes or within an interphase nucleus. The number of chromosomes in the interphase nucleus is determined by counting the number of hybridization regions.

In these studies, we have used one conventional approach (the mouse micronucleus assay) and two molecular cytogenetic techniques (FISH with chromosome-specific probes for mouse chromosomes 8 and 14 and the modified micronucleus assay using tandem probes) to detect and characterize chromosomal alterations occurring in bone marrow cells of animals administered benzene. The mouse bone marrow erythrocyte micronucleus assay is well known and has been the subject of a number of extensive reviews (MacGregor et al 1987; Mavournin et al 1990). For additional background, the reader is referred to those sources. Because the FISH assays are less commonly used, however, a brief description of each of these assays follows.

For the tandem labeled mouse micronucleus assay, the origin of micronuclei induced in bone marrow erythrocytes is identified by hybridizing the cells with the mouse major and minor satellite probes and determining whether the centromeric region of a chromosome is present within a micronucleus. The basis for the assay is shown in Figure 2. For this assay two different DNA probes are used. The mouse major satellite probe hybridizes to repetitive DNA sequences in the centromeric heterochromatin adjacent to the long arm of mouse chromosomes, whereas the mouse minor satellite probe targets an adjacent centromeric region that is linked to the telomere of the short arm (Horz

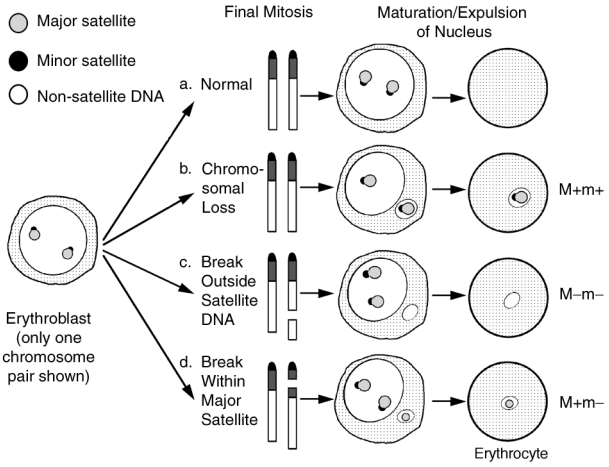

Figure 2. Different mechanisms leading to the formation of micronuclei in the mouse in vivo bone marrow micronucleus assay and the expected results obtained by multicolor FISH with mouse major and minor satellite probes. (a) Normal final division in the developing erythrocyte with expulsion of the main nucleus where a micronucleus is not present in the erythrocyte. (b) Chromosomal loss during the final mitosis resulting in a micronucleus that contains an entire chromosome. Following FISH, hybridization regions for both the mouse major and minor satellite will be present (M+m+). (c) An acentric fragment originating from breakage outside the mouse major and minor satellite regions. In this case, the resulting micronucleus will show no hybridization signal (M−m−). (d) A micronucleus originating from a chromosomal fragment formed from breakage in the region targeted by the major satellite probe. Following FISH, a hybridization region for the major satellite, but not the minor satellite, will be present in the resulting micronucleus (M+m−). A micronucleus containing only the minor satellite sequence (M−m+) is infrequently seen and is probably due to a hybridization artifact (see text for explanation).

and Altenburger 1981; Narayanswami et al 1992; Pardue and Gall 1970). These probes hybridize to the centromeric region of 39 of the 40 mouse chromosomes: the Y-chromosome does not contain either type of satellite DNA and is not labeled by these probes. Using this assay, micronuclei with a number of different origins can be identified: micronuclei containing both the major (M) and minor (m) satellite probes (M+m+) indicate that the micronucleus originated from loss of an entire chromosome; micronuclei containing only the major satellite signal (M+m−) indicate that the micronucleus was formed as a result of a break within the mouse heterochromatin, a breakage-prone region in mouse chromosomes; and micronuclei failing to hybridize with either the major or minor satellite probes (M−m−) are formed from breakage within the mouse euchromatin. Micronuclei labeling with only the minor satellite probe (M−m+) are uncommon and are probably the result of inadequate hybridization of the major satellite probe. (For an M−m+ micronucleus to be formed, two infrequent events—chromosome loss and a break between the regions targeted by the two probes—would have to take place.) Previous studies have shown that this assay is effective in identifying the origin of micronuclei formed by either chromosome loss or breakage within the mouse heterochromatin or within the mouse euchromatin (Chen and Eastmond 1995a; Chen et al 1994b; Grawe et al 1997).

The second assay uses FISH with chromosome-specific DNA probes to detect changes in chromosome number that have occurred in the bone marrow cells of the treated mice (Eastmond and Pinkel 1990; Eastmond et al 1995). A schematic diagram of this assay is shown in Figure 3. This assay is essentially the same whether conducted in mouse or human cells. In this study, the assay was performed on both mononucleated and polymorphonucleated mouse bone marrow cells. Fewer chromosome-specific probes have been developed for the mouse, and the signals are typically weaker and more diffused than those seen in human cells. The signals for the chromosome 8 and 14 probes used in this study, although adequate for interphase FISH, were often somewhat diffuse because the targeted regions are comprised of interspersed repeat sequences. For a variety of technical reasons, this assay is much more effective at detecting increases in chromosome number (hyperdiploidy and polyploidy) than it is in detecting chromosome loss (see Eastmond and Pinkel 1990; Eastmond et al 1995 for more detailed discussions). The hyperdiploidy referred to throughout this report refers to nuclei containing three or more hybridization regions and includes polyploid cells as well as aneuploid cells with additional chromosomes. Because only a single probe

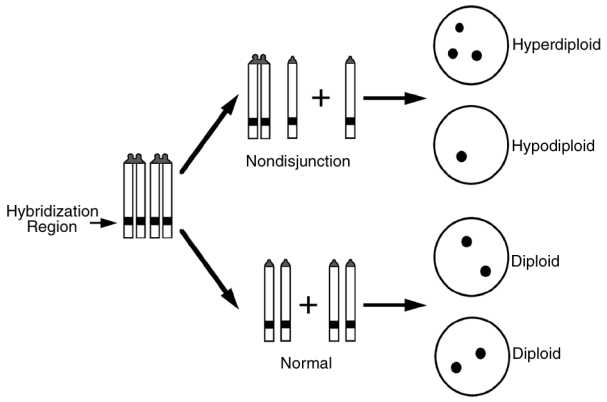

Figure 3. Schematic representation of FISH with chromosome-specific DNA probes to detect hyperdiploidy and hypodiploidy in nucleated bone marrow cells. Bottom: Following a normal mitosis, both daughter nuclei will contain 2 copies of the chromosome of interest and should exhibit 2 hybridization signals for that particular chromosome. Top: In the case of a nondisjunction event, one daughter nucleus will contain 1 (or 0) hybridization signal, whereas the other daughter nucleus will be hyperdiploid with 3 (or 4) copies of the chromosome of interest. After FISH with a chromosome-specific DNA probe, one nucleus should show 1 (or 0) hybridization signal and the other nucleus 3 (or 4) hybridization signals.

was used at a time, a distinction between these two related types of numerical aberrations could not be made.

MATERIALS AND METHODS

Animals

Male B6C3F$_1$ mice were obtained from Charles River Laboratories (Raleigh NC) at 8 weeks of age. Mice were acclimated for 1 week in an Airo-Neg Safety Inclosure (microisolater) maintained in a room with a constant temperature of 21°C to 23°C, a relative humidity of 45% to 54%, and a 12-hour light-dark cycle. The animals were housed randomly at four to six per cage in polycarbonate cages with hardwood-chip bedding and received food (Laboratory Rodent Diet #5001, PMI Feeds, St Louis MO) and water ad libitum.

Chemical Treatment

Animals were dosed 5 days per week following the dosing regimen of the National Toxicology Program's chronic animal bioassay of benzene (National Toxicology Program [NTP] 1986). To assess alterations during a relatively steady state and to avoid a decrease that might occur if sampling occurred immediately following the two days without treatment, the animals were killed toward the end of the last week of treatment. This meant that the animals were killed on days 11, 41, and 82. For simplicity, the reported harvest time for each of the experiments has been rounded to the nearest whole week. Benzene (>99%) was obtained from Aldrich Chemical Company (Milwaukee WI) and mixed with 100% Mazola corn oil by inversion and kept on ice until use. The dosing solutions were prepared fresh daily. In the 2-week study (8 doses over a 10-day period), groups of 6 male mice were administered 0, 50, 100, or 400 mg/kg benzene by oral gavage for 5 days a week, followed by a 2-day period without dosing. Animals were killed 24 hours after the eighth and final dose. For the 6-week (29 doses over a 40-day period) and 12-week (59 doses over an 81-day period) studies, groups of 4 and 10 male mice respectively received 0, 100, or 400 mg/kg benzene for 5 days a week and animals were killed 24 hours after the final dose. One mouse in the control, one in the 100 mg/kg, and two in the 400 mg/kg dose groups died over the course of the 12-week study. At the end of the treatment period, blood was withdrawn by cardiac puncture and bone marrow preparations were made using standard procedures (MacGregor et al 1987). Blood and bone marrow cells were smeared onto slides and fixed in 90% methanol at −20°C for 20 min. Slides were stored desiccated in a nitrogen atmosphere at −20°C until use.

Conventional Micronucleus Assay with Acridine Orange Staining

For staining the bone marrow preparations, the acridine orange method of Hayashi et al (1983) with the following modifications was used: Slides were stained with acridine orange (Sigma, 0.1% stock diluted 1:30 with Sörensen phosphate buffer [pH 6.8]) for 2.5 min at room temperature and rinsed twice for 3 min with phosphate buffer. The preparations were mounted with the same buffer, sealed with rubber cement, and examined for micronuclei within 1 day.

All slides were randomized and coded prior to scoring. Scoring was performed using a Nikon microscope with fluorescence attachment and magnification at ×1,250. For analysis of bone marrow micronuclei, a minimum of 1,000 normochromatic erythrocytes (NCEs) and 1,000 polychromatic erythrocytes (PCEs) were scored for each animal per dose using a blue filter (Nikon B-2A; excitation at 475 to 495 nm, emission at 520 nm). For PCE:NCE ratios, the number of NCEs per 200 PCEs was determined. PCEs, by their orange-red appearance, were easily distinguished from NCEs, which did not show orange-red fluorescence. Micronuclei exhibited a very bright yellowish green fluorescence.

Probes, Probe Generation, and Labeling Conditions

DNA probes hybridizing to the minor satellite sequences of all mouse chromosomes but the α-chromosome were generated by polymerase chain reaction (PCR) using a single 25mer primer for the human α-consensus sequence (Baldini et al 1993) designated as Not Iα 5′-GCG GCC GCC TTC GTT GGA AAC GGG A-3′ (Cruachem, Sterling VA) and DNA isolated by standard methods (Davis et al 1986) from the mouse 3T3 cell line (American Type Culture Collection [ATCC], Rockville MD). For the major satellite probe, we used a 27mer primer for the murine γ-satellite sequence (Vissel and Choo 1989) designated as MGsat-2 5′-CTC TTT ATG TGT GAA ATC CTG CAC-3′ (Cruachem). PCR conditions were similar to those described in Hasegawa et al (1995). After hot-starting the reaction by denaturing the DNA at 94°C for 5 min and then adding 5 U of *Thermus flavus* (*Tfl*) polymerase (Epicenter Technologies, Madison WI), amplification was performed for 30 cycles of 30 sec at 94°C, 30 sec at 42°C, and 1 min at 72°C, followed by one cycle of 15 min at 72°C. PCR amplification products were nick translated according to the protocol provided with the DNA polymerase/DNase enzyme mixture (Amersham, Arlington Heights IL) as described in Hasegawa et al (1995). Digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis IN) for the minor satellite and Cy3-dUTP (Amersham) for the major satellite were used to label the probes in the nick translation reaction.

The mouse chromosome–specific DNA probes for chromosome 8 (4a and 5e) (Boyle and Ward 1992) and chromosome 14 (pL116) (Vourc'h et al 1993) were a generous gift from David Ward (Yale University). Inserts were amplified using oligonucleotide primers for the pBS plasmid and isolated plasmid DNA as template. A 24mer primer named WBS2 5′-CTC GAA ATT AAC CCT CAC TAA AGG-3′ for the T3 promoter region, a 24mer primer designated as WBS4 5′-GAA TTG TAA TAC GAC TCA CTA TAG-3′ for the T7 promoter (Weier et al 1991), and plasmid DNA isolated by standard methods were used. PCR and nick translation conditions were the same as above. Bio-16-dUTP (Boehringer Mannheim) was used to label the probes by nick translation.

Modified Micronucleus Assay with Mouse Minor and Major Satellite Probes

Fluorescence in situ hybridization experiments were performed using modifications of previously described methods (Trask and Pinkel 1990). For the multicolor FISH with mouse minor and major satellite probes, a hybridization method similar to the one described in Chen et al (1994b) was used. Briefly, the bone marrow cells were fixed in 2% paraformaldehyde for 40 sec and washed in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate, pH 7.0) for 5 min at room temperature. Slides were rinsed with double deionized H_2O (ddH_2O) and dehydrated in an ethanol series (70, 85, 100%) for 2 min each at room temperature. After drying the slides with a nitrogen stream, 10 μL of hybridization cocktail was applied to each slide. The hybridization cocktail contained 20 to 100 ng of each of the digoxigenin-labeled minor-satellite and Cy3-labeled major-satellite probes, 1 μg sonicated herring sperm DNA, and 1 μg human blocking DNA in 55% formamide, 10% dextran sulfate, and 1× SSC. Both probe and target DNA were then denatured on a 60°C slide warmer simultaneously and hybridized overnight at 37°C in a humidified chamber. Following hybridization, slides were washed in 50% formamide/2× SSC three times for 5 min each, all at 40°C. The slides were rinsed in PX buffer (0.1 M phosphate buffer, pH 8.0 containing 0.2% Triton-X-100 [Sigma]) for 5 min at room temperature, and the digoxigenin-labeled minor satellite probe was detected using a fluorescein-conjugated sheep anti–digoxigenin antibody (20 μg/mL in PX buffer with 5% nonfat dry milk [PXM], Boehringer Mannheim). DNA was counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (2.5 μg/mL) in a diphenylenediamine antifade mounting medium.

All slides were randomized and coded prior to scoring. A minimum of 2,000 erythrocytes, regardless of their classification as PCEs or NCEs, were scored using a Nikon fluorescence microscope at ×1,250 magnification and a triple-band pass filter (Chroma Technology, Brattleboro VT; #P/N 61002) to visualize simultaneously the yellow-green (fluorescein), red (Cy3) and blue (DAPI). Following the observation of a micronucleus, a blue filter (Nikon B-2A, excitation at 475 to 495 nm, emission at 520 nm) for the yellow-green fluorescein signals (minor satellite probe) and a green filter (Chroma; #31004; excitation at 540 to 580 nm, emission at 600 to 660 nm) for the red Cy3-signals (major satellite probe) were used to verify the presence or absence of each probe.

Fluorescence in Situ Hybridization with Chromosome-Specific DNA Probes

Prior to hybridization, slides were washed in 2× SSC for 5 min and dehydrated in an ethanol series for 2 min each, all at room temperature. Following the application of 10 μL of hybridization cocktail, target DNA and probe were denatured simultaneously for 5 min at 85°C on a slide warmer. The hybridization cocktail consisted of 20 to 100 ng of the biotin-labeled DNA probe for chromosome 14 or 20 to 100 ng each of the biotin-labeled chromosome 8–specific probes (chrom 84 and chrom 85), 1 μg human blocking DNA in 55% formamide, 1× SSC, and 10% dextran sulfate. Following hybridization overnight at 37°C in a humidified chamber, slides were washed in 2× SSC, three times for 5 min each in 50% formamide/2× SSC and once in 2× SSC, all at 46°C. Slides were rinsed in PX buffer for 5 min at room temperature, and 20 μL of fluorescein isothiocyanate (FITC)–avidin (5 μg/mL in PXM; Vector Laboratories, Burlingame CA) was used to detect the biotinylated probe DNA. Propidium iodide (0.5 μg/mL) in diphenylenediamine antifade was used to counterstain the DNA.

All slides were randomized and coded prior to scoring. A minimum of 1,000 mononuclear and 1,000 polymorphonuclear bone marrow cells were scored using a Nikon fluorescence microscope at ×1,250 magnification and a blue filter (Nikon B-2A, excitation at 475 to 495 nm, emission at 520 nm) to visualize the yellow-green fluorescein signals and orange propidium iodide counterstain simultaneously. Mononuclear bone marrow cells were distinguished by their round appearance from polymorphonuclear cells, which have irregular shapes or lobular structures. Cells with three or four hybridization regions for the chromosome of interest were classified as hyperdiploid for that particular chromosome.

Statistical Analyses

The micronucleus and hyperdiploidy data were analyzed using linear regression analysis, or analysis of variance (ANOVA) on the square root ($x + 0.5$)–transformed

data, or both (Lovell et al 1989, 1991). Regression was used to identify dose-related effects, whereas ANOVA was used to determine whether significant differences had occurred within the experiment. Protected Fisher least significant difference (PFLSD) was used as a post hoc test to identify significant differences between individual treatments. Critical values were determined using a 0.05 level of type I error.

RESULTS

A series of three related but separate studies were conducted to assess the contribution of various types of chromosomal alterations occurring in mouse bone marrow following benzene administration for 2 weeks, 6 weeks, or 12 weeks. For each animal, chromosomal damage was initially assessed using the erythrocyte micronucleus assay. Second, the origin of the micronuclei was determined using the major and minor satellite probes. This allowed erythrocyte micronuclei originating from chromosome loss, breakage within the mouse heterochromatin, and breakage within the euchromatin to be distinguished. Last, FISH with probes specific for subcentromeric regions of mouse chromosomes 8 and 14 was used to identify the frequency of hyperdiploidy occurring in the mononuclear and polymorphonuclear cells of the mouse bone marrow. The results from each of the assays at each time point are presented in the following sections.

Short-Term Exposure to Benzene (2 Weeks)

Bone Marrow Erythrocyte Micronucleus Assay A strong dose-related increase in the frequency of micronuclei was detected in the newly formed PCEs of the benzene-treated mice (Figure 4). The frequency of micronuclei in the PCEs increased from 4 (± 2.7)‰ in the controls to 29.8 (± 7.7)‰ in the animals treated with the 400 mg/kg dose of benzene. The frequency of micronuclei in the PCEs was significantly increased at all doses compared with the control frequencies ($P \leq 0.05$, PFLSD). A similar dose-related increase, although of a lower magnitude, was seen in the older micronucleated NCEs (Figure 4). The frequency of micronuclei in the NCEs was significantly increased at the two highest doses with frequencies ranging from 2.8 (± 1.2)‰ in the controls to 10.3 (± 6.4)‰ in the mice treated with the highest benzene dose ($P \leq 0.05$, PFLSD). Somewhat surprisingly, no change in the PCE:NCE ratio was seen (Table 1), probably reflecting the high variability that was seen at this time point.

DNA Probe Assay on Micronucleated Erythrocytes Multicolored FISH with the mouse major and minor satellite probes was used to classify the erythrocyte micronuclei and identify their origins. In the previous assay, staining of the erythrocytes with acridine orange allowed the newly formed PCEs to be distinguished from the older NCEs. In the probe assay, however, DAPI was used as a DNA counterstain, which does not allow PCEs to be differentiated

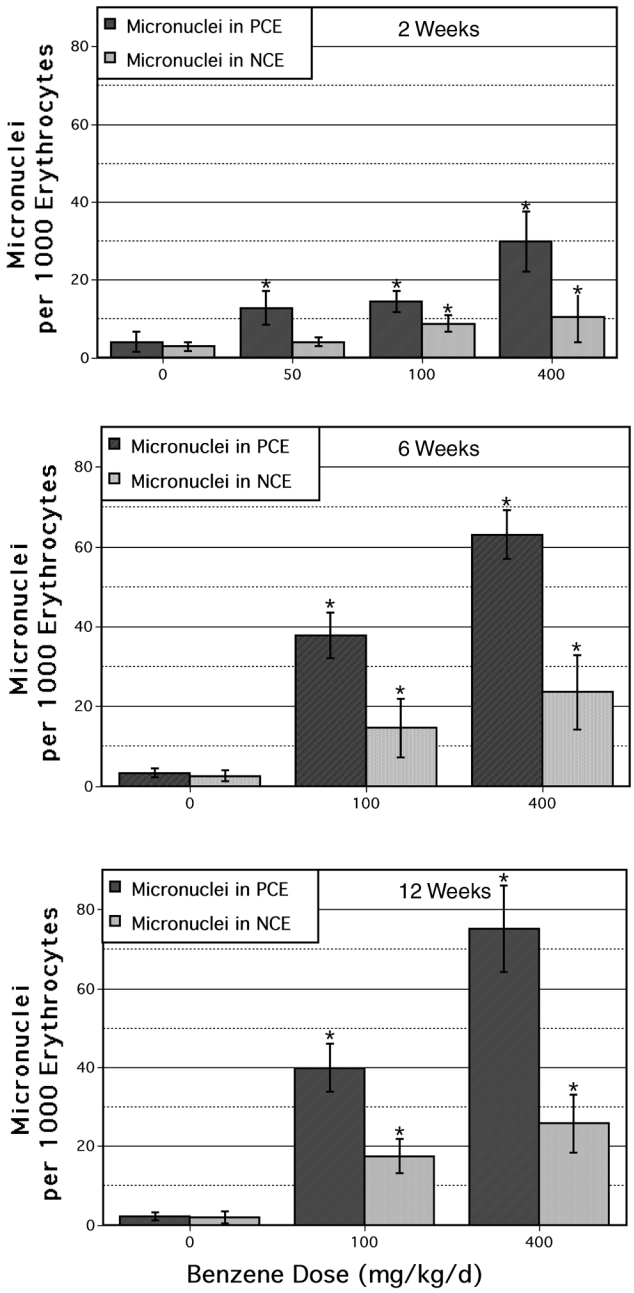

Figure 4. Induction of micronuclei in bone marrow erythrocytes of B6C3F$_1$ mice untreated or treated for 2, 6, or 12 weeks with the indicated dose of benzene (acridine orange staining). The frequency of micronuclei was determined by scoring 1,000 PCEs and 1,000 NCEs. An asterisk (*) indicates a significant difference from untreated animals ($P \leq 0.05$; PFLSD).

Table 1. Ratio of Polychromatic to Normochromatic Erythrocytes in the Bone Marrow of Control and Benzene-Treated $B6C3F_1$ Mice[a]

Duration of Exposure (weeks)	Benzene Exposure (mg/kg/day)			
	0	50	100	400
2	1.06 ± 0.29	1.08 ± 0.36	0.85 ± 0.32	0.97 ± 0.50
6[b]	1.32 ± 0.37	—	0.68 ± 0.37[c]	0.44 ± 0.29[c]
12[b]	1.16 ± 0.39	—	0.76 ± 0.32[c]	0.50 ± 0.07[c]

[a] Values are presented as means ± SD.

[b] Significant dose-related decreases in the PCE:NCE ratio were seen in the 6- and 12-week studies ($P \leq 0.05$; regression analysis).

[c] Differs significantly from its respective control ($P \leq 0.05$; PFLSD).

from NCEs. As a result, the frequency of micronuclei are reported for all erythrocytes. As seen in the previous assay, a clear increase in total erythrocyte micronuclei was observed (Figure 5). The frequency of micronuclei increased from 7.5 (± 2.6) per 2,000 erythrocytes in the controls to 28.7 (± 10.9) per 2,000 in the animals treated with the highest dose of benzene. This increase was due to increases in micronuclei originating from chromosome loss (M+m+) as well as micronuclei formed as a result of breakage within the mouse heterochromatin (M+m−) and the mouse euchromatin (M−m−). No increase was seen in M−m+ micronuclei; these micronuclei occur infrequently and probably are the result of inefficient hybridization or detection, rather than indicating micronuclei containing only the minor satellite probe. The frequency of micronuclei originating from chromosome loss (M+m+) increased from 2.8 (± 1.7) per 2,000 erythrocytes in the controls to 14.8 (± 3.9) per 2,000 erythrocytes at the highest dose—a 5.3-fold increase. The frequency of M+m− micronuclei, those originating from breakage in the mouse heterochromatin, increased 5.4-fold from 0.8 (± 1.7) per 2,000 erythrocytes to 4.3 (± 2.3) at the 400 mg/kg dose. The frequency of M−m− micronuclei, indicating micronuclei originating from breakage in the mouse euchromatin, increased from 3.8 (± 1.2) per 2,000 erythrocytes to 24.3 (± 7.3) at the highest dose, representing a 6.4-fold increase.

DNA Probe Assay on Bone Marrow Mononuclear and Polymorphonuclear Cells Numerical chromosome alterations in the mononuclear and polymorphonuclear cells of the bone marrow of control mice and benzene-treated mice were evaluated using FISH with DNA probes specific for subcentromeric regions on mouse chromosomes 8 and 14. Using either the chromosome 8 or chromosome 14 probes (Table 2), the frequency of hyperdiploidy in the bone marrow cells remained close to control frequencies across all doses of benzene. For example, the frequency of hyperdiploidy for chromosome 8 was 1.8 (± 0.7)‰, 3.3 (± 1.5)‰, 1.9 (± 1.4)‰, and 3.4 (± 1.6)‰ for the controls and the 50, 100, and 400 mg/kg doses, respectively. Although there was a suggestion of a dose-related increase, the results were variable and the increase did not attain statistical significance ($P = 0.08$). No increase was seen in the frequency of hypodiploid mononuclear cells using the chromosome 8 probe ($P = 0.72$). Similar results were seen when using the chromosome 14 probe. In this case, however, the hyperdiploid increase attained statistical significance ($P = 0.014$). The frequencies of hyperdiploidy for chromosome 14 were 2.6 (± 1.6)‰, 4.1 (± 1.6)‰, 2.7 (± 1.1)‰, and 5.4 (± 2.1)‰ for the controls and the 50, 100, and 400 mg/kg doses, respectively. The frequency of hyperdiploidy 14 in the mononuclear cells at the high dose was significantly higher than that observed in the controls ($P \leq 0.05$).

Similar results were obtained in the analyses of the polymorphonuclear leukocytes for both the chromosomes 8 and 14 probes. The frequency of hyperdiploidy ranged from 1.6‰ and 2.4‰ in the controls and increased to 2.6‰ and 3.2‰ at the highest dose for chromosomes 8 and 14, respectively. Again, the frequencies of hyperdiploidy exhibited variability across doses, and the trends were not statistically significant. This variation was probably due in part to the relatively small numbers of samples and of cells scored per sample. By combining the results from the two probes and combining the results from the two types of cells, it was possible to increase the accuracy of the cytogenetic measurements. With the pooled data, a relatively weak, but statistically significant, dose-related increase in the frequency of hyperdiploid cells was seen (Table 2, $P \leq 0.05$, regression). The effect was modest, however, increasing from approximately 2‰ (8.4 per 4,000 cells) in the controls to less than 4‰ (14.5 per 4,000 cells) in animals at the highest dose.

For both the analyses using the chromosome 8 and 14

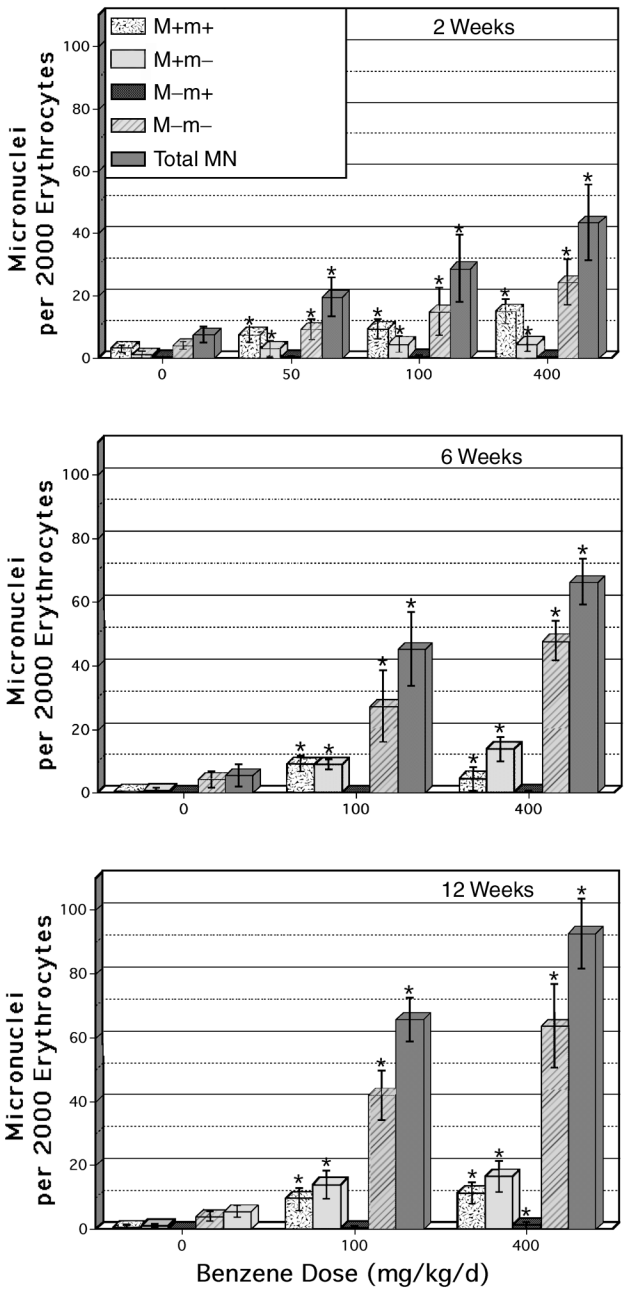

Figure 5. Induction of micronuclei in bone marrow erythrocytes of B6C3F$_1$ mice untreated or treated for 2, 6, or 12 weeks with the indicated doses of benzene (FISH). The frequency of micronuclei was determined by scoring 2,000 erythrocytes following multicolor FISH with mouse major and minor satellite probes. An asterisk (*) indicates a significant difference from untreated animals ($P \leq 0.05$; PFLSD). MN = micronuclei.

probes, the percentage of mononuclear and polymorphonuclear cells on the slides was recorded as a measure of benzene's effects on the various cell lineages. An examination of these data indicated that there was a significant decrease in the percentage of mononuclear cells with dose (Table 2). The percentage of mononuclear cells decreased from 60% in the controls to 51% at the high dose, a statistically significant difference ($P \leq 0.05$; PFLSD).

Mid-Term Exposure to Benzene (6 Weeks)

To assess the chromosomal effects of benzene at an intermediate point between the 2-week and the 12-week studies, four animals at each dose were killed at 6 weeks and cytogenetic analyses were performed using each of the assays described above. Due to the strong nature of the effects in the micronucleus assay and the weak effects seen in the aneuploidy assay, it was decided to study only the 100 mg/kg and the 400 mg/kg doses at the 6-week and 12-week time points.

Bone Marrow Erythrocyte Micronucleus Assay As seen in the 2-week study, clear dose-related increases in micronuclei were seen in the bone marrow PCEs of the benzene-treated mice (Figure 4). The frequency of micronuclei increased from 3.3 (± 1.5)‰ in the controls to 63 (± 6.1)‰ at the 400 mg/kg dose. A significant increase in micronuclei was also seen in the NCEs, increasing from 2.5 (± 1.3)‰ in the controls to 23.5 (± 9.3)‰ at the highest dose. In addition, a significant decrease in the ratio of PCEs to NCEs was seen (Table 1). The PCE:NCE ratio decreased from 1.32 (± 0.37) in the controls to 0.44 (± 0.29) at the 400 mg/kg benzene dose.

DNA Probe Assay on Micronucleated Erythrocytes Probes for the mouse major and minor satellite probes were used to identify the origin of the induced micronuclei. As in the 2-week study, a clear dose-related increase in the frequency of total micronuclei was seen, with micronucleus frequencies increasing from 5.5 (± 3.4) per 2,000 erythrocytes in the controls to 66.3 (± 7.1) per 2,000 at the high dose (Figure 5). Again, the increase in micronuclei at this time point was due to both chromosomal loss and chromosomal breakage, as increases in M+m+, M+m−, and M−m− micronuclei were seen. The increases in M+m+ micronuclei, although elevated at the 100 and 400 mg/kg doses ($P \leq 0.05$; PFLSD), did not exhibit a consistent increase with increasing dose. Total micronuclei increased 12-fold whereas the contributing three classes of micronuclei increased 9-fold, 18-fold, and 11-fold, respectively, when comparing the frequencies in mice treated at the high dose with those of the controls.

DNA Probe Assay on Bone Marrow Mononuclear and Polymorphonuclear Cells Chromosome-specific DNA probes for mouse chromosomes 8 and 14 were used to assess numerical alterations in the benzene-treated and control animals. As before, analyses using the individual

Table 2. Frequency of Nuclei Exhibiting Hyperdiploidy for Chromosomes 8 and 14 in the Mononuclear and Polymorphonuclear Cells in Bone Marrow from Control and Benzene-Exposed B6C3F$_1$ Mice[a]

	Mononuclear or Polymorphonuclear Cells	Benzene Exposure (mg/kg/day)			
		0	50	100	400
2 Weeks					
Chromosome 8	Mono	1.8 ± 0.7	3.3 ± 1.5	1.9 ± 1.4	3.4 ± 1.6
	PMN	1.6 ± 0.8	2.1 ± 1.1	1.9 ± 1.5	2.6 ± 2.6
Chromosome 14	Mono[b]	2.6 ± 1.6	4.1 ± 1.7	2.7 ± 1.1	5.4 ± 2.2[c]
	PMN	2.4 ± 0.8	4.2 ± 2.5	2.6 ± 2.4	3.2 ± 2.2
Chromosomes 8 and 14	Mono	4.3 ± 2.0	7.4 ± 2.5	4.6 ± 2.1	8.8 ± 1.8
	PMN	4.0 ± 1.6	6.3 ± 2.5	4.5 ± 2.7	5.7 ± 2.6
	Mono + PMN[b]	8.4 ± 1.8	13.6 ± 4.3[c]	9.1 ± 1.8	14.5 ± 3.2[c]
Percentage of Mononuclear Cells[d]		*60% ± 4%*	*59% ± 3%*	*57% ± 5%*	*51% ± 6%*[c]
6 Weeks					
Chromosome 8	Mono	4.3 ± 3.0	—	6.0 ± 3.4	7.0 ± 1.4
	PMN	2.8 ± 1.5	—	6.5 ± 4.4	6.3 ± 2.5
Chromosome 14	Mono	4.0 ± 1.8	—	6.8 ± 1.5	5.3 ± 1.5
	PMN[b]	4.0 ± 1.8	—	3.5 ± 1.0	6.8 ± 2.8
Chromosomes 8 and 14	Mono	8.3 ± 2.8	—	12.8 ± 3.3	12.3 ± 0.5
	PMN	6.8 ± 3.1	—	10 ± 4.8	13.0 ± 4.2
	Mono + PMN[b]	15.0 ± 5.4	—	22.8 ± 6.5	25.3 ± 4.6[c]
Percentage of Mononuclear Cells[d]		*43% ± 4%*	—	*35% ± 2%*[c]	*38% ± 4%*
12 Weeks					
Chromosome 8	Mono	2.1 ± 1.5	—	2.4 ± 2.3	2.5 ± 1.6
	PMN	1.8 ± 1.1	—	4.1 ± 1.5[c]	2.5 ± 1.5
Chromosome 14	Mono	4.8 ± 2.9	—	5.0 ± 4.4	7.5 ± 2.4
	PMN[b]	3.5 ± 1.6	—	4.7 ± 3.5	6.9 ± 3.0[c]
Chromosomes 8 and 14	Mono	7.1 ± 2.5	—	6.9 ± 3.4	10.3 ± 1.7
	PMN	5.4 ± 2.4	—	8.6 ± 4.2	9.6 ± 4.0
	Mono + PMN[b]	12.5 ± 4.6	—	15.4 ± 7.3	19.9 ± 4.3[c]
Percentage of Mononuclear Cells[d]		*45% ± 4%*	—	*35% ± 5%*[c]	*33% ± 7%*[c]

[a] Mice were exposed to indicated levels of benzene for 2, 6, or 12 weeks. Hyperdiploidy was defined as cells having three or more hybridization signals. Frequency was determined per thousand cells.

[b] A significant dose-related increase in hyperdiploidy was seen across all doses ($P \leq 0.05$; regression analysis).

[c] Value differs significantly from the respective control value ($P \leq 0.05$; PFLSD).

[d] Percentage of mononuclear cells in bone marrow white blood cells. A significant dose-related decrease in the percentage of mononuclear cells was seen across all doses ($P \leq 0.05$; regression analysis).

probes failed to detect significant increases in hyperdiploidy in the bone marrow of the treated animals. The frequencies of hyperdiploidy in the mononuclear and polymorphonuclear cells using the chromosome 8 and 14 probes are shown in Table 2. As seen in the 2-week study, the frequency of hyperdiploidy in the cells from the treated animals (generally 5‰ to 7‰) was somewhat variable but typically higher than that seen in the controls (approximately 3‰ to 4‰).

After combining the data from the two probes and the

two cell types, a weak but statistically significant dose-related increase in hyperdiploidy was seen ($P \leq 0.05$; Table 2). The frequency of hyperdiploidy increased from 15 (± 5.4) per 4,000 cells to 25.3 (± 4.5) per 4,000 cells, indicating that benzene treatment did cause a modest increase in the frequency of hyperdiploid cells. Somewhat surprisingly, a slight increase in the frequency of hypodiploid cells was seen with increasing doses of benzene ($P = 0.053$). The percent of mononucleated cells was modestly decreased in the benzene-treated animals (Table 2). However, the decrease was only significant at the 100 mg/kg dose ($P \leq 0.05$).

Longer-Term Exposure to Benzene (12 Weeks)

To determine the effects of longer-term exposure to benzene, cytogenetic studies were conducted on animals exposed to 0, 100, and 400 mg/kg benzene for 12 weeks. The results of each of the assays conducted are presented below.

Bone Marrow Erythrocyte Micronucleus Assay As seen at the two earlier time points, a strong dose-related increase in the frequency of micronuclei was seen in the bone marrow PCEs of the benzene-treated mice (Figure 4). The frequency of micronuclei increased from 2.1 (± 1.1)‰ in the controls to 75 (± 10.9)‰ at the 400 mg/kg dose. A similar increase in micronuclei was also seen in the NCEs, increasing from 1.9 (± 1.5)‰ in the controls to 25.8 (± 7.3)‰ at the highest dose. In addition, a significant decrease in the ratio of PCEs to NCEs was also seen (Table 1) with the PCE:NCE ratio decreasing from 1.16 (± 0.39) in the controls to 0.50 (± 0.07) at the 400 mg/kg dose of benzene.

DNA Probe Assay on Micronucleated Erythrocytes The micronuclei formed in the bone marrow erythrocytes were characterized using probes for the mouse major and minor satellite regions. As seen in both the 2-week and 6-week studies, a clear dose-related increase in the frequency of total micronuclei was seen, with micronucleus frequencies increasing from 5.4 (± 1.9) per 2,000 erythrocytes in the controls to 92.6 (± 11.0) per 2,000 at the high dose (Figure 5). As seen in the previous studies, the increase in micronuclei at this time point was due to both chromosome loss as well as breakage within the mouse heterochromatin and euchromatin, as increases were seen in each of the M+m+, M+m−, and M−m− classes of micronuclei. A minor increase was also seen in M−m+ micronuclei, primarily due to an elevated frequency at the highest dose. As indicated above, this type of micronuclei is believed to result from inefficient hybridization or detection. Total micronuclei increased 17-fold whereas the contributing three classes of micronuclei increased 26-fold, 17-fold, and 16-fold, respectively, when comparing the frequencies in mice treated with the high dose with those of the controls. At the highest benzene dose, chromosome breakage was responsible for approximately 87% of the total micronuclei.

DNA Probe Assay on Bone Marrow Mononuclear and Polymorphonuclear Cells Using the chromosome-specific DNA probes for mouse chromosomes 8 and 14, the frequency of hyper- and hypodiploidy was assessed in the mononuclear and polymorphonuclear cells of the bone marrow of benzene-treated and control mice (Table 2). Again, analyses using the individual probes generally failed to detect significant increases in hyperdiploidy or hypodiploidy in the individual bone marrow cells of the treated animals. Occasionally a significant association was seen for one chromosome in one cell type. A similar increase was not seen for the other chromosome or in the other cell type, however. For example, a significant dose-related increase in hyperdiploidy was detected using the chromosome 14 probe in the polymorphonuclear cells. A similar increase was not seen using the chromosome 8 probe, nor was a significant increase seen for chromosome 14 in the mononuclear cells. In each case, however, the frequency in the treated mice was slightly elevated above that seen in the controls. As indicated above by combining the data from both probes and both cell types, a more accurate measure of hyperdiploidy was achieved. When this was performed, a significant increase in the frequency of hyperdiploid cells was seen in the benzene-treated animals compared with controls (Table 2). The frequency increased from 12.5 (± 4.6) per 4,000 cells to 19.9 (± 4.3) per 4,000 cells, indicating that benzene exposure resulted in a modest but significant increase in the frequency of hyperdiploid cells in the mouse bone marrow. Interestingly, benzene treatment was also associated with a significant increase in the frequency of hypodiploid cells. As seen at the earlier time points, a significant dose-related decrease in the percentage of mononuclear cells in the mouse bone marrow was seen with increasing doses of benzene (Table 2). Of the bone marrow cells, 45% consisted of mononuclear cells in the control mice compared with 35.3% and 32.5% mononuclear cells, which were seen at the 100 and 400 mg/kg benzene doses, respectively.

DISCUSSION

A compilation of the key results from the three studies is shown in Figures 6 and 7. By comparing the results of the three experiments, a number of trends related to the

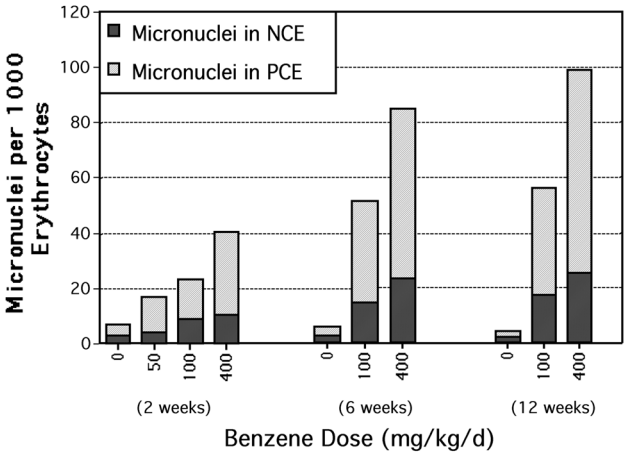

Figure 6. Compilation of results over time for the induction of micronuclei in bone marrow erythrocytes of B6C3F$_1$ mice untreated or treated for 2, 6, or 12 weeks with the indicated dose of benzene (acridine orange staining). The frequency of micronuclei was determined by scoring 1,000 PCEs and 1,000 NCEs. Note that the frequency of PCEs is added on top of the frequency of NCEs in each bar.

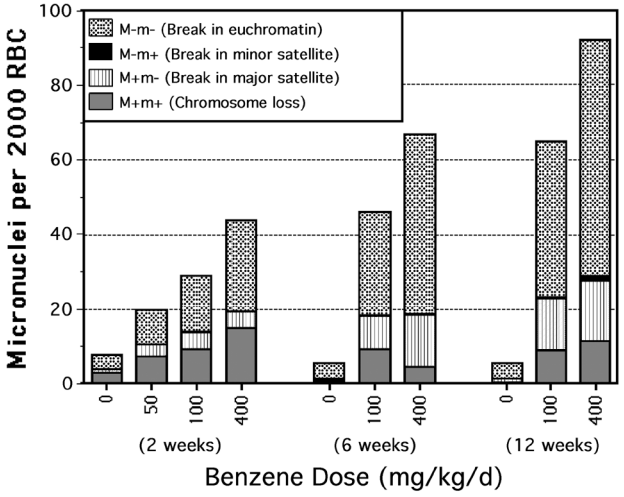

Figure 7. Compilation of results over time for the induction of micronuclei in bone marrow erythrocytes of B6C3F$_1$ mice untreated or treated for 2, 6, or 12 weeks with the indicated doses of benzene (FISH). The frequency of micronuclei was determined by scoring 2,000 erythrocytes following multicolor FISH with mouse major and minor satellite probes. Note that the frequency for each micronucleus class is added on top of each other class.

individual experiments and duration of benzene exposure can be seen. Consistent and strong dose-related increases in the frequency of micronuclei were seen at each of the time points. These increases were due primarily to chromosome breakage, although significant dose-related increases in chromosome loss were also seen. Across the three studies, it is apparent that the frequency of micronuclei induced by benzene increased with increasing duration of exposure. This was confirmed in the statistical analyses, in which a strong significant association between micronucleus frequency and benzene dose as well as a benzene $\times$ time interaction was seen. In contrast, there was no significant change in the micronucleus frequencies in the control animals with time. The strong increase in micronuclei that occurred in the PCEs is particularly interesting in that this increase represents damage occurring in newly formed cells rather than an accumulation of damage over time. As indicated, the increase appeared to be largely due to chromosome breakage, particularly that occurring within the mouse euchromatin. These results indicate that the frequency of chromosome breakage increases in the benzene-treated animals over time, suggesting either a shift in the metabolite profile resulting in the formation of more clastogenic metabolites or possibly an increase in genomic instability in the treated mouse erythroblasts with time.

Weak but consistent increases in hyperdiploidy were observed at all three time points when the results of the two cell types and two probes were combined. The inability to detect significant increases using the results of the individual assays is probably due to a weak effect combined with relatively high variability. By combining the assay results, the estimates of hyperdiploid frequency became more accurate and allowed the relatively weak effect to be seen. Although a slight increase in hyperdiploidy occurred with increasing duration of exposure, the magnitude of the increases observed in the benzene-treated animals was modest at all time points. Similar experiments conducted in our laboratory during the same time period using the chromosome 8 and 14 probes demonstrated that significant increases in hyperdiploidy could be readily detected in the bone marrow of mice treated with vincristine sulfate, a model aneuploidy-inducing agent. The results of these experiments indicate that hyperdiploidy is induced by benzene, but that the frequency of aberrant cells exhibiting abnormal numbers of a specific chromosome is quite low.

Although these studies were cytogenetic in nature and not specifically designed to look for bone marrow toxicity, we measured several endpoints that indicated that significant cellular damage occurred in the bone marrow of the benzene-treated mice. The PCE:NCE ratio, commonly used in the micronucleus assay as an indicator of bone marrow toxicity, showed significant decreases at the 6-week and 12-week time points, reflecting a treatment effect on erythrocyte formation or maturation. In addition, a significant benzene-related decrease in the percentage of mononuclear cells in the bone marrow was seen at each time point. A decrease in the percentage of mononuclear cells was also seen with time. The first effect is an indication of benzene-related alterations in the bone marrow. The second is prob-

ably related to changes in the composition of bone marrow cells that occur with age in rodents (Valli et al 1990).

At one or two time points, an increase in the frequency of cells exhibiting zero and one hybridization regions was observed in the bone marrow cells of the benzene-treated animals. Although this decrease in hybridization signals may represent a true change in chromosome number, in our experience this endpoint can be quite variable in untreated cells and is highly influenced by hybridization conditions. (For additional discussion, see Eastmond and Pinkel 1990; Eastmond et al 1995.) The chromosome 8 and 14 probes used in this experiment, although adequate for aneuploidy detection using FISH, consist of interspersed repeats and, as a consequence, are more diffuse than the probes that are typically used to detect aneuploidy in human cells. As a result of this combination of factors, we place considerably less weight on the hypodiploidy endpoint than on those measuring micronuclei or hyperdiploidy.

These studies have shown that both aneuploidy and chromosome breakage are induced in the bone marrow of mice following administration of high doses of benzene. Many types of chemicals have been shown to induce aneuploidy in mammalian cells (Eastmond 1993; Oshimura and Barrett 1986). In most cases, these agents are thought to interact with protein targets, such as the mitotic spindle, within the cell. A number of years ago, Irons and associates postulated that the quinone metabolites of benzene may act as spindle poisons by disrupting mitotic assembly during cell division (Irons 1985; Irons et al 1981, 1984). Subsequent studies by these investigators and others have shown that the benzene metabolites hydroquinone and 1,4-benzoquinone are inhibitors of microtubule assembly in isolated microtubule preparations and in mouse lymphocytes in vitro (Epe et al 1990; Irons 1985; Irons et al 1984; Pfeiffer and Metzler 1996). These studies provide a plausible mechanism to explain the alterations in chromosome number seen in the benzene-treated animals.

A number of mechanisms have been proposed that might explain the increase in chromosome breakage seen in animals and humans following benzene exposure. These include binding of benzene metabolites to DNA, the generation of reactive oxygen species and subsequent adduct formation, and an interference of benzene with enzymes involved in DNA replication or repair. Numerous studies have shown that a number of the reactive benzene metabolites are capable of binding to DNA in vitro (Pongracz and Bodell 1991; Pongracz et al 1990; Reddy et al 1990; Schatz-Kornbrust et al 1991; Snyder et al 1987). These results have been supported by animal studies in which DNA binding has been detected in vivo following the administration of benzene (Creek et al 1997; Lutz 1979; Lutz and Schlatter 1977; Norpoth et al 1988; Pathak et al 1995). The magnitude of the covalent binding recovered following benzene administration is low, however, and rankings of carcinogenic agents by DNA binding ability generally rank benzene among the weakest of initiating agents, approaching those that act through indirect genotoxic mechanisms (Creek et al 1997; Lutz 1986; Reddy et al 1990).

The generation of oxygen radicals and derived adducts have been observed in vivo following benzene exposure (Subrahmanyam et al 1991). In addition, increased levels of superoxide dismutase as well as lipid peroxidation have been detected in the bone marrow of benzene-treated mice, suggesting that elevated levels of free radicals as well as reactive oxygen species such as hydrogen peroxide (H_2O_2) are generated during benzene metabolism (Khan et al 1984; Pandya et al 1986, 1989). These results are supported by reports of elevated levels of 8-hydroxydeoxyguanosine in the bone marrow of benzene-treated mice (Kolachana et al 1993) as well as in the urine of workers exposed to benzene-containing petroleum products (Lagorio et al 1994; Nilsson et al 1996). Time-course studies performed by Kolachana et al (1993), however, indicated that the oxygen radical–derived adducts disappeared rapidly from bone marrow, presumably due to efficient repair of this type of adduct. 8-Hydroxydeoxyguanosine adducts were detected within minutes of benzene administration, but within 1 to 2 hours they had been eliminated from the DNA and the adduct levels had returned to control levels. Additional in vitro studies have also shown that reactive oxygen species are formed during autoxidation of the hydroquinone and 1,2,4-benzenetriol and may contribute to the cytotoxic and clastogenic effects of these agents (Dobo and Eastmond 1994; Irons et al 1982; Lewis et al 1988). These results indicate that reactive oxygen species are formed following benzene exposure and could be involved in the clastogenic effects of benzene. Whether these reactive oxygen species contribute directly to benzene's clastogenic effects or contribute indirectly through the formation of H_2O_2 and a stimulation of peroxidase-mediated bioactivation remains an area of uncertainty.

The relatively weak binding of benzene to DNA and the rapid repair of oxygen radical–derived adducts, combined with benzene's potent clastogenic effects, have led some investigators to investigate the role of proteins and enzymes involved in DNA replication and repair as potential targets for benzene's reactive metabolites. In vivo studies that have used radiolabeled benzene to investigate binding have measured substantially more radiolabel bound to bone marrow proteins than to DNA (Arfellini et al 1985; Creek et al 1997; Mazzullo et al 1989). Other researchers have investigated the inhibitory effects of

benzene and its metabolites on specific enzymes involved in DNA replication and cell homeostasis. These studies have shown that various quinone and quinone-forming metabolites of benzene are capable of inhibiting DNA and RNA polymerases, providing evidence for the potential involvement of protein targets in benzene clastogenicity (Lee et al 1989; Post et al 1984; Schwartz et al 1985).

In summary, the results of these experiments indicate that FISH techniques can be successfully used to detect both chromosome breakage and aneuploidy resulting from chemical exposure. B6C3F_1 mice administered benzene exhibited both aneuploidy and breakage. Chromosome breakage was the predominant effect, and this occurred primarily within the mouse euchromatin. Significant breakage within the mouse heterochromatin was also observed, as was aneuploidy. The aneuploidy resulting from benzene exposure in mice is a relatively infrequent event, with increases in both chromosome loss and hyperdiploidy being seen.

TOPOISOMERASE INHIBITION*

Although benzene is widely recognized to induce chromosomal aberrations in both humans and animals, the mechanisms underlying its clastogenic effects and their relationship to leukemogenesis remain unknown. As indicated previously, studies of this agent have shown that benzene exhibits weak binding to DNA (Lutz 1986) and is weakly mutagenic or nonmutagenic in most gene mutation assays (IPCS 1993; Waters et al 1988). In addition, oxygen radical–derived adducts formed following benzene exposure appear to be repaired very rapidly (Kolachana et al 1993).

The mechanisms by which benzene exerts its genotoxic effects in the bone marrow appear to be complicated, involving multiple metabolites and molecular targets. This is supported by evidence that the coadministration of various benzene metabolites—including phenol, hydroquinone, and catechol—result in a potentiation of cytotoxicity and genotoxicity in the bone marrow of treated mice (Eastmond et al 1987; Guy et al 1990; Marrazzini et al 1994). Research from our laboratory and others has recently shown that a synergistic increase in micronuclei is observed following the combined treatment of phenol and hydroquinone in mice (Barale et al 1990; Chen and Eastmond 1995a; Marrazzini et al 1994). This increase was largely the result of an increase in chromosome breakage within the euchromatic region of the mouse chromosomes (Chen and Eastmond 1995a). Based on the differential repair capacities of the heterochromatic and euchromatic DNA (Mellon et al 1986), we proposed that the increased chromosomal breakage within the euchromatin might be due to an inhibitory effect of benzene's phenolic metabolites on enzymes involved in DNA replication and repair.

For a number of years it has been recognized that certain agents can induce chromosomal aberrations through interactions with non-DNA targets such as DNA polymerases (van Zeeland et al 1982), DNA ligase (Jha et al 1992), and poly(ADP-ribose) polymerase (Vanni et al 1998; Yager and Wiencke 1997), indicating a role for enzymes and proteins involved in DNA replication and repair in clastogenesis. Recently, a new class of human leukemia–inducing agents, the epipodophyllotoxins, has been identified in clinical trials (Pedersen-Bjergaard and Philip 1991; Pedersen-Bjergaard and Rowley 1994). These clastogenic compounds exert their clastogenic and leukemogenic effects through interaction with topoisomerase II rather than through covalent binding to DNA (Pedersen-Bjergaard and Rowley 1994).

Topoisomerase II enzymes relieve torsional strain on DNA that occurs during replication and transcription by creating transient breaks in both strands of double-stranded DNA and allowing the passage of a second DNA strand (Ferguson and Baguley 1994). These enzymes are also important structural components of interphase nuclei and are believed to function during recombination, chromosome condensation, and DNA repair (Downes et al 1991; Ferguson and Baguley 1994; Stevnsner and Bohr 1993). Interference with normal topoisomerase II activity at critical stages of the cell cycle can lead to chromosome breakage, aneuploidy, or cell death.

A number of topoisomerase II inhibitors are quinone or quinone-forming compounds that exhibit structural similarity to the metabolites of benzene that possess hydroxyl or keto groups on the aromatic ring (D'Arpa and Liu 1989; Frydman et al 1997; Gantchev and Hunting 1997, 1998; Kim et al 1996; Skibo et al 1997). We therefore hypothesized that the quinone and phenolic metabolites of benzene might exert their clastogenic effects through inhibition of topoisomerase enzymes. Furthermore, quinone-forming phenolic compounds have been identified at relatively high concentrations in the peripheral blood and bone marrow of rodents following benzene exposure (Rickert et al 1981). Based on these observations, we initiated a series of studies to determine whether benzene and its metabolites could exert their hematopoietic effects through an inhibition of topoisomerase enzymes in the bone marrow.

* When published, the studies of benzene and its metabolites binding to topoisomerase II will include K Turteltaub and J Vogel as coauthors.

MATERIALS AND METHODS

Chemicals and Enzymes

Phenol, hydroquinone, catechol, 1,4-benzoquinone, 1,2,4-benzenetriol, 2,2′-biphenol, and 4,4′-biphenol (all ≥ 98% purity) were purchased from Aldrich Chemical (Milwaukee WI). Glutathione (GSH reduced form, 98% to 100%), H_2O_2 (30%), and horseradish peroxidase (HRP) type VI (250 U/mg) were obtained from Sigma Chemical (St Louis MO). Deionized distilled water was purchased from Mallinckrodt Chemical (Paris KY). *t,t*-Muconaldehyde was a generous gift of Dr Gisela Witz (Rutgers University, Piscataway NJ). Human topoisomerase I, II, catenated kinetoplast DNA (kDNA), supercoiled plasmid substrate DNA, teniposide (also known as VM26), *m*-amsacrine, and anti–human topoisomerase II-α polyclonal antibody were purchased from TopoGEN (Columbus OH). Protease inhibitors were obtained from Boehringer Mannheim (Mannheim, Germany). Protein A–agarose beads were purchased from Calbiochem (Cambridge MA). Immuno-enhanced chemiluminescence (ECL-plus) Western blotting analysis system reagents and anti–rabbit HRP–linked whole antibodies were acquired from Amersham Life Science (Arlington Heights IL). [U-^{14}C]Phenol (> 99%; specific activity of 40 mCi/mmol) was purchased from ICN Biomedicals (Irvine CA). [^{14}C]4,4′-Biphenol (nominal purity > 98%; 15.4 mCi/mmol) was acquired from Sigma Chemical Company (St Louis MO). [^{14}C]Hydroquinone (nominal purity 98%; 22 mCi/mmol) was purchased from Wizard Laboratories (Davis CA). All other chemicals, including those for the synthesis of diphenoquinone, were obtained from Sigma Chemical.

2,2′-Biphenol, 4,4′-biphenol, and *m*-amsacrine were dissolved in 100% dimethyl sulfoxide (DMSO) at 100 mM concentrations, with subsequent dilutions in 1% DMSO. *t,t*-Muconaldehyde was dissolved and diluted in 100% ethanol. Phenol, hydroquinone, catechol, 1,4-benzoquinone, and 1,2,4-benzenetriol were prepared in ddH_2O. Chemical concentrations are reported as final concentrations in the total assay volume. The initial concentration to which the enzyme was exposed varied depending on the order in which the reaction components were added.

Bioactivation Using High Peroxidase and Hydrogen Peroxide Conditions

Dilutions of HRP, a model peroxidase enzyme, and the 30% H_2O_2 stock were made in ddH_2O immediately before the reaction. The final enzymatic activity in the reaction for HRP was 0.08 U and the final concentration for H_2O_2 was 500 mM. Stock solutions of phenol, hydroquinone, catechol, 1,4-benzoquinone, and 1,2,4-benzenetriol were prepared in ddH_2O. 2,2′-Biphenol and 4,4′-biphenol were dissolved in 100% DMSO at 100 mM. All subsequent dilutions to the tested concentrations were made in ddH_2O. All the compounds were tested at three or more concentration points. The reactions were performed in 1.5-mL microcentrifuge tubes. The reaction volumes were 20 μL containing 0.08 U HRP and 500 μM H_2O_2. After the initiation of the reaction, the reaction mixture was incubated at room temperature for 1 hour before testing in the topoisomerase inhibition assay. For the studies employing GSH, reduced GSH at 100 μM was added to the reaction mixture 1 hour after its initiation. Following a 10-min incubation at room temperature, the reaction mixtures were tested for inhibitory activity in the topoisomerase II assay without further purification.

Peroxidase-Mediated Metabolic Bioactivation Using Reduced Oxidizing Conditions

For these studies, several peroxidase activation conditions were used. For the initial titration studies and the binding study, the assay conditions consisted of 0.8 U/mL HRP and 500 μM H_2O_2 with the reaction proceeding for 1 hour. For the balance of the assays using phenol and 4,4′-biphenol, enzyme and H_2O_2 concentrations in the incubations were 0.07 U/mL HRP and 55 μM H_2O_2. In these latter studies, 2,2′-biphenol was bioactivated with 0.1 U/mL HRP and 55 μM H_2O_2 to facilitate more complete metabolism. Peroxidase bioactivation reactions were run for 5 min at room temperature with the exception that the 2,2′-biphenol incubations were performed for 30 min. At the end of the reaction period, the incubations were placed on ice, at which time the assay buffer, kDNA, and topoisomerase II enzyme were added sequentially for the topoisomerase assay.

In Vitro Topoisomerase I Inhibition Assays

The topoisomerase I assay was performed according to the protocol provided by TopoGEN. The reactions contained 0.25 μg supercoiled (Form 1) pHOT1 plasmid DNA, the assay buffer (10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl; pH 7.5), 5 U human topoisomerase I, and the benzene metabolite to be tested. Incubations were performed for 30 min at 37°C, following which the supercoiled and open circular DNAs were resolved by electrophoresis using a 1% agarose gel. All compounds were tested a minimum of two times.

In Vitro Topoisomerase II Inhibition Assays

The testing of actual and putative benzene metabolites was done using a commercially available topoisomerase II

inhibition assay (TopoGEN). The kit included purified human topoisomerase II (2 U/μL), catenated kDNA (0.1 μg/μL), and 10× assay buffer (0.5 M Tris-Cl, pH 8.0; 1.2 M potassium chloride [KCl], 100 mM magnesium chloride, 5 mM adenosine triphosphate [ATP], 5 mM dithiothreitol, and 300 μg/mL bovine serum albumin [BSA]). Assays were performed in the presence and absence of test chemicals or solvents (1 μL added to assay) by mixing 4 U enzyme with 0.2 μg kDNA and 3 μL 10× assay buffer. The reaction was brought to a final volume of 30 μL with deionized distilled water (0.2 μm filtered). For the standard assay method, the order of addition to the assay was H_2O, 10× assay buffer, followed by either the test compound, the solvent, or the metabolic reaction mixture, kDNA, and topoisomerase II. For the direct method, the metabolite or the reaction mix was added directly to the topoisomerase II, followed by H_2O, 10× assay buffer, and kDNA. The incubations were run for 1 hour at 37°C and the reaction terminated by addition of 6 μL of a stop solution consisting of 5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol in H_2O. In the initial studies, 100 μM teniposide was used as the positive control, whereas *m*-amsacrine (3 mM) was used in the later studies. The DNA products, as well as catenated kDNA standard or decatenated marker kDNA were separated by electrophoresis using a 1% agarose gel and 1× TAE buffer containing 0.03 μg/mL ethidium bromide. The DNA-containing bands were visualized using an ultraviolet light box and photographed using Polaroid type 57 film. All experiments were repeated and any chemicals that exhibited an inhibition of topoisomerase II were tested again to verify the results.

Titration Assays

Titration assays of phenol, 2,2′-biphenol, and 4,4′-biphenol assays were performed in the presence of 100 μM peroxidase-activated phenol, 2,2′-biphenol, 4,4′-biphenol, or solvents (1 μL added to assay) by mixing either 4 U of enzyme with increasing amounts of kDNA for the DNA titrations or 0.2 μg of kDNA with increasing amounts of topoisomerase II for the enzyme titration assays. The reaction mixture also contained 3 μL 10× assay buffer, with the reaction being brought to a final volume of 30 μL with deionized distilled water (0.2 μm filtered). For these assays, the order of reagent addition was H_2O, 10× assay buffer, followed by the peroxidase metabolic reaction mixture, kDNA, and topoisomerase II. The enzyme and kDNA were incubated for 1 hour at 37°C, and the reaction was terminated and electrophoresis was performed as described above. The experiments were repeated three times.

Diphenoquinone Synthesis

Diphenoquinone was synthesized using the method of Konig and coworkers (1960). Briefly, the synthesis was performed by adding 7.14 g lead tetracetate to 140 mL glacial acetic acid. Concurrently, 2 g 4,4′-biphenol was added to 80 mL anhydrous 1,4-dioxane. Over 2 min, the 4,4′-biphenol solution was added to the lead tetracetate solution and stirred for 5 min. The red-brown particles formed were filtered off using a glass frit filter and then recrystalized from acetone. Melting point and UV analysis of the crystals agreed with previously published results (Konig et al 1960). The melting point of the crystals was 220°C, and UV analysis of the crystals dissolved in chloroform showed a characteristic peak at 396 nm (Konig et al 1960). The structure was also verified by high-resolution mass spectrometry, using a VG 7070 high-resolution mass spectrometer with a desorption direct-insertion probe and ammonia carrier gas. Analysis by mass spectrometry was done at the University of California, Riverside, mass spectrometry facility.

Binding Studies of Isolated Topoisomerase II

The experiments were designed to test the binding activity of [^{14}C]phenol or [^{14}C]phenol metabolites to topoisomerase II and other proteins (Table 3). [^{14}C]Phenol was converted to its reactive metabolites by HRP as described above. Nonradioactive phenol (97.2 μM) and 2.8 μM [^{14}C]phenol were mixed to reach a final concentration of 100 μM of phenol in the reaction. A total concentration of 100 μM for phenol was chosen for this study because a strong inhibition of topoisomerase II was observed at this concentration in our previous assays. After a 1-hour incubation with HRP, 10× topoisomerase II assay buffer and topoisomerase II were added to the reaction products as illustrated in the experimental set-up (Table 3). The reactions were incubated for 15 min at 37°C followed by a 10% sodium dodecyl sulfate (SDS) treatment for 15 min at 37°C. For the GSH conjugation assay, 100 mM GSH was added to the HRP reactions 10 min before topoisomerase II. The concentration of proteins was measured using the Coomassie Blue method of Sedmak and Grossberg (Sedmak and Grossberg 1977). Amounts of the proteins in the reactions were 40 and 400 ng for topoisomerase II and HRP, respectively.

Protein Separation: In Vitro Binding Studies

The proteins in the reaction mixture were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN II cell (Bio-Rad Laboratories, Hercules CA) at 100 V. Proteins in the gel were visualized by silver staining (Silver Staining Plus kit, Bio-Rad Laboratories).

Table 3. Experimental Design for the Studies of [^{14}C]Phenol Metabolites Binding to Topoisomerase II and Horseradish Peroxidase in Vitro

Reaction Number[a]	Objective	Topoisomerase II	[^{14}C]Phenol	Horseradish Peroxidase	Reduced Glutathione
1	Topoisomerase II binding	+	+	+	−
2	Horseradish peroxidase binding	−	+	+	−
3	Reduced glutathione effect on binding	+	+	+	+
4	Blank control	+	−	+	−
5	[^{14}C]Phenol control[b]	+	+	−	−

[a] $n = 3$ assays for each reaction. A + indicates the chemical was added to the reaction mixture.

[b] Without peroxidase-mediated bioactivation.

Each protein band on the gel representing topoisomerase II or HRP was excised and stored in microcentrifuge tubes. The gel slices were weighed to ensure that each slice contained a similar wet mass. Background ^{14}C radioactivity, whether due to natural ^{14}C occurrence or [^{14}C]phenol or its HRP-mediated metabolites tailing through the gel during electrophoresis, was accounted for by excising the corresponding area on the gel in lanes where either [^{14}C]phenol or topoisomerase II or HRP was not present.

Before analysis by accelerator mass spectrometry (AMS), the amount of radioactivity in a duplicate experiment was checked using liquid scintillation counting against background to ensure that the sample had not exceeded the maximum radioactivity of approximate 1.4 dpm/mg of gel slice as suggested by Vogel (1992). The gel slices were dried under vacuum in silica tubes and converted to graphite using the process described by Vogel and associates (1987, 1989). ANU sucrose (prepared by the Australian National University), with an activity 1.508 times the ^{14}C activity of 1950 carbon, was converted to graphite along with the samples to monitor for ^{14}C carryover and was used as an analytical standard.

Accelerator Mass Spectrometry Measurements

The AMS analysis was performed in collaboration with investigators at the Center for Accelerator Mass Spectrometry at the Lawrence Livermore National Laboratory, Livermore CA. Measurements were recorded in units of modern and calculated as [^{14}C]phenol or [^{14}C]phenol equivalents (in femtomoles) per protein (in picomoles). One modern is defined as 0.0979 fmol of ^{14}C atoms per milligram of carbon and is approximately equal to the natural abundance of ^{14}C present in contemporary (1950 AD) carbon (Stuiver and Polach 1977). Sample measurements were performed by using the protocols developed for the AMS beamline at the Lawrence Livermore National Laboratory (Davis 1989; Proctor 1989).

Human HL-60 Cell Culture

Human HL-60 cells (ATCC, Rockville MD) were grown in RPMI 1640 (Media Tech, Washington DC) supplemented with 20% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana CA), 2 mM L-glutamine, 100 IU/mL penicillin, and 10 mg/mL streptomycin at 37°C in a 5% CO_2 environment and not used beyond 35 passages.

Dose-Response Cytotoxicity Studies in Human HL-60 Cells

Approximately 1×10^7 total cells in 20 mL of complete media (5×10^5 cells/mL) were exposed to concentrations ranging from 0 to 1,000 μM phenol, catechol, 1,2,4-benzenetriol, hydroquinone, 2,2′-biphenol, or 4,4′-biphenol in DMSO. The final concentration of DMSO in the culture media did not exceed 0.1% of the total volume. Hydrogen peroxide was supplemented at levels between 0 and 20 μM to mimic more closely the metabolic conditions believed to be present in the bone marrow during toxicity. Cells were grown as described above to an approximate density of 5×10^5 cells/mL, then continuously exposed to the chemical for 48 hours. Samples were taken at 2, 4, 8, 24, and 48 hours and viability determined by trypan blue dye exclusion (Freshney 1994). To determine cell viability, 1 mL of cell suspension (approximately 1 mL, 5×10^5 total cells) was removed from the primary culture flask, centrifuged at 1,200 rpm for 5 min, and the supernatant removed. The cells were resuspended in 1 mL Hanks balanced salt solution (HBSS) lacking Ca^{++} and Mg^{++}; 100 μL of a 0.4% trypan blue dye solution was added to the suspension and vortexed. Using an inverted light microscope, 100 cells were evaluated for the presence of trypan blue; cells

excluding the dye were counted as being viable. All experiments were performed a minimum of three times. The results of the cytotoxicity studies were used to estimate doses at which topoisomerase II inhibition would be likely to occur.

Cellular Studies of Topoisomerase II Enzyme Activity

Approximately 1×10^8 total cells in 200 mL of complete media (5×10^5 cells/mL) were exposed to either 500 μM 4,4′-biphenol for 8 hours, or 50 μM hydroquinone, 500 μM catechol, or 100 μM benzenetriol for 2 hours. Chemicals were dissolved in 100% DMSO and added to the cell culture media. The final concentration of DMSO in the culture media did not exceed 0.1% of the total volume. The 4,4′-biphenol, hydroquinone, and catechol exposures were supplemented with 10 μM H_2O_2 in an attempt to increase the peroxidase activation of the compounds. Cell viability was determined by trypan blue exclusion at the end of the exposure period. [^{14}C]4,4′-Biphenol or [^{14}C]hydroquinone binding studies in HL-60 cells were performed in the same manner as above with either 5 μCi of [^{14}C]4,4′-biphenol (15.4 mCi/mmol; 500 μM total dose) or 2 μCi of [^{14}C]hydroquinone (22 mCi/mmol; 50 μM total dose) being added to the cell culture.

Topoisomerase II was extracted using the method of Gieseler and colleagues (1994, 1996). An equal number of viable cells were used for nuclear protein extractions for each concentration and control, and all steps were performed on ice. Between 5×10^7 and 1×10^8 viable cells were centrifuged at 4°C, the supernatant removed, and the cells resuspended in 7.5 mL lysis buffer (0.3 M sucrose, 0.5 mM EGTA [pH 8.0], 60 mM KCl, 15 mM NaCl, 15 mM Hepes [pH 7.5], 150 μM spermine, and 50 μM spermidine). Buffer containing Triton X-100 (40 μL Triton X-100 per 500 μL lysis buffer, warmed to 37°C) was added to the cell suspension and inverted four times. The tubes were incubated on ice for 15 min and occasionally inverted to prevent the cells from settling. The suspension was centrifuged at 1,200 rpm ($300 \times g$) for 5 min at 4°C. The supernatant was removed and the pellet resuspended in 0.5 mL lysis buffer. The nuclear suspension was then centrifuged for 5 min at 3,000 rpm ($1{,}875 \times g$) in a microfuge tube and the supernatant removed. The pellet containing the nuclear suspension was resuspended at a concentration corresponding to 3×10^7 nuclei/mL in extraction buffer (5 mM potassium phosphate [pH 7.5], 100 mM NaCl, 1 μL/mL 14.3 M 2-mercaptoethanol, and 5 μL/mL 200 mM phenylmethylsulfonyl fluoride [PMSF] dissolved in 100% DMSO); 5 M NaCl was slowly added to the suspension to make a final volume of 10% (v/v). Following this, the tubes were gently shaken to lyse the nuclei. The suspension was then centrifuged at $15{,}000 \times g$ for 10 to 15 min to remove the DNA. The supernatant was centrifuged a second time to ensure that all the DNA was removed. The protein concentration of the extract was determined using the method described by Sedmak (Sedmak and Grossberg 1977). Extracts were then assayed for topoisomerase II activity as described above. Gel photographs were scanned using an Epson 636 scanner and the amount of decatenated kDNA or relaxed plasmid DNA was quantified by gray scale analysis using the public domain NIH Image Program 1.61 Gel Plotting Macro. All experiments were replicated a total of three times.

Studies of Topoisomerase Inhibition in Mouse Bone Marrow

Male B6C3F$_1$ mice 6 weeks of age were obtained from Charles River Laboratory (Wilmington MA). Between 15 to 25 male B6C3F$_1$ mice were administered 440 mg/kg benzene in approximately 200 μL corn oil by oral gavage daily for 3 days. The mice were killed 24 hours following the final dose. Mouse bone marrow was extracted by flushing 1 mL RPMI 1640 through each femur and pooling the bone marrow extracts from all animals in a dose group. The bone marrow was centrifuged at 1,200 rpm ($300 \times g$) for 10 min and the supernatant removed. To lyse the erythrocytes, the concentrated cells were resuspended in 1 mL RPMI 1640 plus 10 mL freshly made 0.85% ammonium chloride. This suspension was allowed to stand on ice for 10 min. The suspension was then centrifuged, the supernatant removed, and the cells resuspended in 10 mL fresh media. The nucleated cells were counted using a hemocytometer and the viability determined by trypan blue dye exclusion. Topoisomerase II was extracted from an equal number of viable cells and the extracts were assayed for topoisomerase II activity as described previously. [^{14}C]Benzene binding studies were performed using the above methods, with 440 mg/kg [^{14}C]benzene (1.6 mCi/mmol) administered to mice. Based on previous AMS studies, significant binding of radioactive benzene to bone marrow proteins has been seen at similar doses (Creek et al 1997).

In Vivo Topoisomerase II Protein Levels and ^{14}C Chemical Protein Binding Studies

The topoisomerase II protein levels and [^{14}C]benzene binding to topoisomerase II in mouse bone marrow were examined by first extracting nuclear proteins from nucleated bone marrow cells as previously described. We next immuno-precipitated the proteins using the methods outlined below. The proteins were then separated by SDS-PAGE and the protein transferred to polyvinylidenedifluoride (PVDF) membrane for ECL-plus detection. After

visualizing the detectable protein standards and mouse bone marrow proteins (not detectable), the bands corresponding to molecular weights of interest were excised and radiocarbon levels were determined by AMS.

Immunoprecipitation

Topoisomerase II was extracted as described above with the addition of protease inhibitors to both the lysis buffer and extraction buffers. Protease inhibitors were added at the following concentrations: 74 μM antipain-dihydrochloride, 130 μM bestatin, 50 μM chymostatin, 1.4 μM E-64, 1 μM leupeptin, 1 μM pepstatin, 7 μM phosphoramidon, 4 mM pefabloc, 500 μM EDTA-Na_2, and 0.3 μM aprotinin. Topoisomerase II was immunoprecipitated from nuclear extracts on an equal total protein basis by adding 10 μL of anti–human topoisomerase II antibody (TopoGEN) to the crude nuclear extract and incubating it for 1 hour on a rotating mixer at 4°C. Protein A–agarose beads (50 μL) were then added to the suspension and incubated for 1 hour at 4°C. Beads were concentrated by centrifuging for 10 min at 15,000 × *g* and then resuspended and washed three times in NET buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Igepal, 1 mM EDTA, 0.02% sodium azide, and 1 mM PMSF). The immunoprecipitated mouse nuclear proteins adsorbed to the beads were extracted with 50 μL 2× sample buffer (120 mM Tris [pH 6.8], 4% SDS, 20% glycerol, and 0.05% bromophenol blue) plus 5% β-mercaptoethanol (v/v) and heated to 100°C for 5 min. Proteins were separated by electrophoresis and detected by ECL-plus using the methods described in the next section.

We employed AMS in an effort to measure the low levels of ^{14}C estimated to be associated with topoisomerase II in mouse bone marrow. The methods and procedures used to prepare samples for measurement by AMS were similar to those developed for previous AMS studies and have been outlined previously (Creek et al 1994; Vogel et al 1997).

Cellular Topoisomerase II Protein Levels and Protein Binding Studies

Briefly, topoisomerase II protein levels and [^{14}C]4,4′-biphenol or [^{14}C]hydroquinone binding to topoisomerase II in HL-60 cells were examined by first extracting nuclear proteins from HL-60 cells. The proteins were then separated by SDS-PAGE using the methods of Laemmli (1970). The protein was then transferred to a PVDF membrane for ECL-plus detection per the manufacturer's instructions. After determining the protein levels, the protein bands were excised and ^{14}C levels were determined by scintillation counting. Accelerator mass spectrometry was not used in the cell studies because the higher amounts of radioactivity used could potentially contaminate the instrument.

Protein Separation and Detection

Topoisomerase II was extracted as described above except protease inhibitors were added to both the lysis and extraction buffers. Protease inhibitors were added at the following concentrations: 74 μM antipain-dihydrochloride, 130 μM bestatin, 50 μM chymostatin, 1.4 μM E-64, 1 μM leupeptin, 1 μM pepstatin, 7 μM phosphoramidon, 4 mM pefabloc, 500 μM EDTA-Na_2, and 0.3 μM aprotinin. Equal amounts of protein from the HL-60 cells were separated by 0.1% SDS-PAGE (Laemmli 1970). Protein transfers were done using an X-Cell mini-gel system (Novex, San Diego CA). Proteins were separated using a 3% polyacrylamide stacking gel on top of a 7.5% polyacrylamide separating gel in running buffer (25 mM Tris [pH 8.3], 192 mM glycine, and 0.1% SDS). Electrophoresis was performed at 125 V (30 to 40 mA) for approximately 2 to 4 hours. The gel was soaked in the transfer buffer for 5 min prior to transfer. The proteins were next transferred onto a PVDF blotting membrane (Applied Biosystems) (Towbin et al 1979). Transfers were run for ≥ 8 hours in 12 mM Tris-base, 96 mM glycine, 10% MeOH, and 0.01% SDS at 33 V (140 mA), with the transfer buffer being changed every 2 to 3 hours. The membrane was blocked overnight (≥ 12 hours) in TBS-T (10 mM Tris [pH 8], 150 mM NaCl, and 0.1% Tween 20) with 5% nonfat dried milk. The membrane was washed twice in TBS-T for 5 min. The membrane was incubated for 1 hour with a 1:1,000 dilution of polyclonal rabbit anti–human topoisomerase II (TopoGEN) primary antibody in TBS-T with 5% nonfat dried milk at room temperature. The membrane was then washed in TBS-T twice for 5 min and twice for 15 min. The membrane was incubated for 1 hour with a 1:500 dilution of peroxidase-conjugated anti–rabbit IgG secondary antibody. The membrane was again washed in TBS-T twice for 5 min and twice for 15 min and developed using ECL-plus Western blotting detection system and ECL hyperbond film per the manufacturer's instructions (Amersham, Arlington Heights IL).

The protein bands were then excised from the blotting membrane and the ^{14}C levels measured by scintillation counting on a Beckman Instruments (Irvine CA) liquid scintillation counter (model LS 3801).

Statistical Analyses

The differences in the binding activity of [^{14}C]phenol equivalents to topoisomerase II and HRP proteins were determined using ANOVA. Following a significant result

in the ANOVA, PFLSD test was used post hoc to compare the individual treatments.

Topoisomerase II enzyme activity for the treated samples, as determined by image analysis and expressed as a percentage of control activity, was analyzed for differences from control enzyme activity using a one-group *t* test (StatView SE+Graphics, Abacus Concepts, Berkeley CA, 1987). The mean percent inhibition from each of the three or four separate experiments was used to test for differences between the exposed cells or animals and the controls. The cell viability of control and exposed groups was compared using a Student *t* test. Statistical significance for all analyses was determined using a 0.05 probability of type I error.

RESULTS

Inhibitory Effects of Benzene Metabolites on Human Topoisomerase I

A series of benzene's phenolic metabolites were screened for inhibitory effects on human topoisomerase I in the presence and absence of peroxidase activation. No inhibitory effects were seen under our test conditions for any of the individual compounds or reaction products (Table 4).

Inhibitory Effects of Individual Benzene Metabolites on Human Topoisomerase II

The activity of human topoisomerase II was assayed by decatenation of kDNA (Marini et al 1980), a catenated network of mitochondrial DNA rings isolated from *Crithidia fasciculata*, and the reaction was monitored by the appearance of 2.5 kilobase DNA monomers in either the open circular or relaxed form following gel electrophoresis. The appearance of the open circular or linearized kDNA in the gel indicates an active and functional enzyme (Figure 8). If inhibition occurs, the kDNA remains in the catenated form and does not migrate from the well (TopoGEN 1994).

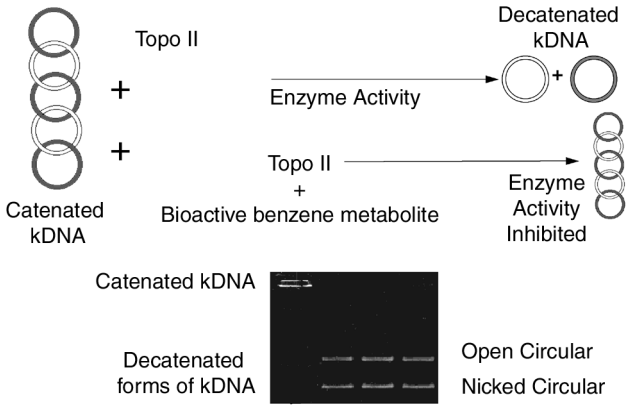

Figure 8. Schematic illustration of the in vitro topoisomerase II assay. In the presence of functional topoisomerase II, catenated kDNA is converted to its decatenated form consisting of open circular and relaxed circular kDNA. These can be distinguished by differential migration through a gel during electrophoresis. In the presence of a topoisomerase inhibitor, decatenated DNA is not formed.

Phenol, hydroquinone, catechol, 1,2,4-benzenetriol, and 1,4-benzoquinone were tested for inhibitory activity on isolated human topoisomerase II. No inhibitory effects were observed for phenol, hydroquinone, or catechol at concentrations as high as 500 μM (Figure 9, lanes

Table 4. Inhibitory Effects of Various Benzene Metabolites on Topoisomerase I

Metabolite	Concentration (μM)	Inhibition[a]
Phenol	1,000	–
Catechol	1,000	–
Hydroquinone	1,000	–
1,4-Benzoquinone	1,000	–
1,2,4-Benzenetriol	1,000	–
Phenol + HRP/H_2O_2[b]	1,000	–

[a] A minus (–) signifies that the electrophoretic pattern of the kDNA from the complete reaction, in the presence of this metabolite, was the same as the control; this indicates the agent tested had no inhibitory effect.

[b] Assay was performed following incubation of phenol with HRP (0.25 U/mL) and H_2O_2 (500 μM) for 1 hour.

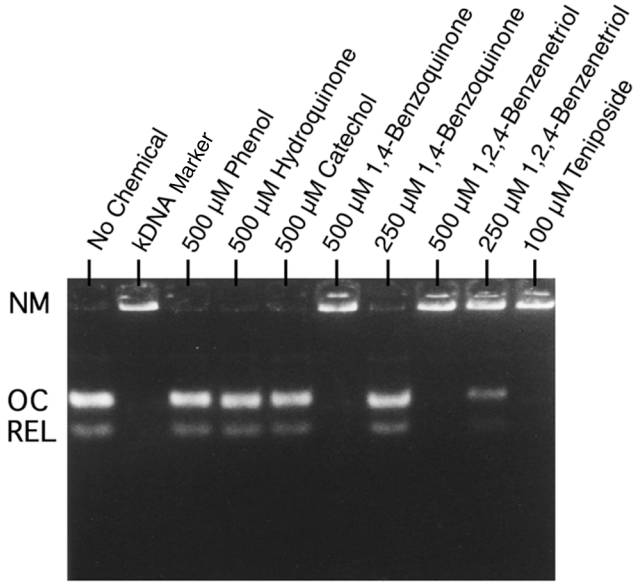

Figure 9. The inhibitory effects of the individual metabolites of benzene on purified human topoisomerase II. NM: kDNA networks exhibiting no migration from the origin; OC: open circular DNA; REL: relaxed DNA. The open circular and relaxed DNA result from topoisomerase II activity allowing the smaller DNA to migrate through the gel.

3, 4, and 5), although inhibition was observed at the 1,000 μM concentrations of hydroquinone and catechol. 1,4-Benzoquinone and 1,2,4-benzenetriol completely inhibited topoisomerase II activity at the 500 μM concentrations (Figure 9, lanes 6 and 8). There was no inhibitory effect at 250 μM for 1,4-benzoquinone (Figure 9, lane 7), however, and only a partial inhibitory effect for 1,2,4-benzenetriol at this concentration (Figure 9, lane 9). Teniposide, a potent inhibitor for topoisomerase II, was used as a positive control in each reaction series (Figure 9, lane 10).

Inhibitory Effects of Phenolic Metabolites on Topoisomerase II in the Presence of a Strong Peroxidase Activation System and the Modifying Effects of Reduced Glutathione

Assays using 2,2′-biphenol and 4,4′-biphenol at 500 μM without bioactivation showed no inhibitory effect on topoisomerase II (Figure 10, lanes 4 and 5). An amount of DMSO equivalent to that added with the 2,2′-biphenol and 4,4′-biphenol solutions was used as a solvent control (Figure 10, lane 6). A complete inhibition of topoisomerase II was observed in reaction mixtures initially containing 100 μM phenol (Figure 10, lane 7) or 2,2′-biphenol (Figure 10, lane 8) following incubation in the presence of a strong peroxidase activation system. Partial inhibitory effects were seen at the 50 μM initial concentration of these two activated compounds. Horseradish peroxidase–activated 4,4′-biphenol completely inhibited the topoisomerase II activity at 50 μM (Figure 10, lane 9) and partially inhibited the enzymatic activity at 10 μM (Figure 10, lane 10). Exclusion of HRP from the reaction mixture eliminated the inhibitory effects on topoisomerase II (Figure 10, lane 11). In addition, no inhibitory effect was observed in the absence of the chemical in the reaction containing both HRP and H_2O_2 (Figure 10, lane 12). The results of the various phenolic metabolites in the topoisomerase II assay are summarized in Tables 5 and 6.

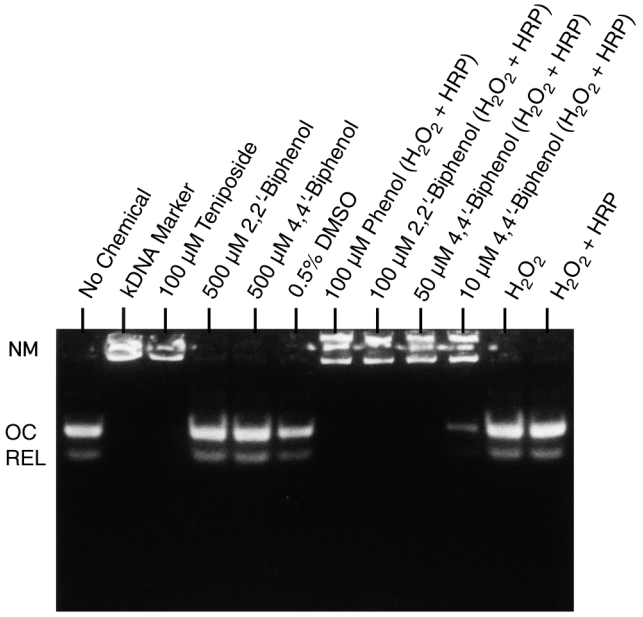

Figure 10. Inhibitory effects of phenol, 2,2′-biphenol, and 4,4′-biphenol on purified human topoisomerase II in the absence and presence of an H_2O_2 + HRP activation system. NM: kDNA networks exhibiting no migration from the origin; OC: open circular DNA; REL: relaxed DNA. The open circular and relaxed DNA result from topoisomerase II activity allowing the smaller DNA to migrate through the gel.

Table 5. Inhibitory Effects of Various Quinone and Phenolic Metabolites of Benzene on Topoisomerase II in the Absence of Peroxidase-Mediated Activation[a]

Metabolite	Concentration (μM)	Inhibition[b]
Phenol	500	−
Catechol	1,000	++
	500	−
Hydroquinone	1,000	++
	500	−
1,4-Benzoquinone	500	++
	250	−
1,2,4-Benzenetriol	500	++
	250	+
	100	−

[a] All assays were repeated and any chemicals exhibiting an inhibition of topoisomerase II were tested again to verify the results.

[b] A minus (−) signifies that the electrophoretic pattern of the kDNA from the complete reaction, in the presence of this metabolite, was the same as the control; this indicates the agent tested had no inhibitory effect.

A + signifies that faint bands representing the kDNA monomers were observed; this indicates the agent tested had a weak inhibitory effect.

A ++ signifies that no visible release of the kDNA monomer from the networked kDNA was observed; this indicates the agent tested had a complete inhibitory effect.

To investigate the chemical properties of the species involved in the inhibition of topoisomerase II, reduced GSH was added to the 4,4′-biphenol reaction containing HRP. The addition of reduced GSH prevented the inhibition of topoisomerase II, suggesting that diphenoquinone or another unidentified reactive intermediate species may be responsible for the inhibition of topoisomerase II (Figure 11, lane 5).

Titration of Topoisomerase II Inhibitors

To investigate the mode of action of phenol metabolites on the inhibition of topoisomerase II, a simple enzyme and DNA titration assay was employed (Tanabe et al 1991).

Table 6. Inhibitory Effects of Various Phenolic Metabolites of Benzene on Topoisomerase II After Peroxidase-Mediated Activation[a]

Metabolite[b]	Metabolite Concentration (μM)	Inhibition[c]
Phenol + HRP/H_2O_2	100	++
	50	+
	10	−
2,2′-Biphenol	500	−
2,2′-Biphenol + HRP/H_2O_2	100	++
	50	−
	10	−
4,4′-Biphenol	500	−
4,4′-Biphenol + HRP/H_2O_2	100	++
	50	++
	10	+
	1	−
4,4′-Biphenol + HRP/H_2O_2 + GSH	100	−

[a] All assays were repeated and any chemicals exhibiting an inhibition of topoisomerase II were tested again to verify the results.

[b] Where HRP/H_2O_2 is indicated, the assay was performed following incubation of the phenolic compound with HRP (0.25 U/mL) and H_2O_2 (500 μM) for 1 hour. Where GSH is indicated, 100 mM GSH was added to the completed peroxidase incubation 10 minutes before adding the topoisomerase reagents.

[c] A minus (−) signifies that the electrophoretic pattern of the kDNA from the complete reaction, in the presence of this metabolite, was the same as the control; this indicates the agent tested had no inhibitory effect.

A + signifies that faint bands representing the kDNA monomers were observed; this indicates the agent tested had a weak inhibitory effect.

A ++ signifies that no visible release of the kDNA monomer from the networked kDNA was observed; this indicates the agent tested had a complete inhibitory effect.

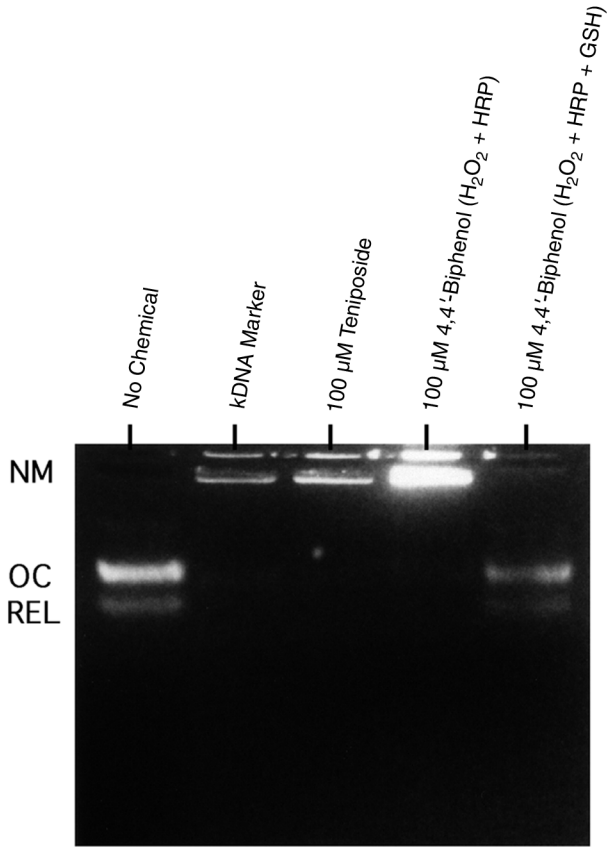

Figure 11. The inhibitory effects of 4,4′-biphenol on purified human topoisomerase II following peroxidase-mediated bioactivation (H_2O_2 + HRP) and the protective effects observed with the addition of GSH. NM: kDNA networks exhibiting no migration from the origin; OC: open circular DNA; REL: relaxed DNA. The open circular and relaxed DNA result from topoisomerase II activity allowing the smaller DNA to migrate through the gel.

Initially, this experiment was performed to determine whether 4,4′-biphenol, the most potent phenolic metabolite identified in previous studies, inhibited topoisomerase II activity by interacting with the enzyme or with DNA. (Similar studies were subsequently conducted for phenol and 2,2′-biphenol as well as 4,4′-biphenol using different activation conditions; see sections below.)

Metabolites formed through the activation of 4,4′-biphenol using strong HRP activation conditions completely inhibited topoisomerase II activity (Figure 12, lane 3). The activity of topoisomerase II was restored, as seen by the release of kDNA into the gel (Figure 12, lanes 4 and 5), when the amount of enzyme in the incubation was increased. Partial recovery of the topoisomerase II activity was observed when 1 U was added to the reaction (Figure 12, lane 4), and complete restoration of enzyme decatenating activity was seen with the addition of 4 U of topoisomerase II (Figure 12, lane 5). In contrast, no protection against inhibition was seen when the amount of kDNA was increased (Figure 12, lanes 6, 7, and 8). These experiments demonstrate that the addition of enzyme, but not DNA, restored enzymatic activity of topoisomerase II following inhibition by bioactivated 4,4′-biphenol, indicating that an interaction of the metabolites with topoisomerase II, rather than DNA, was responsible for the observed enzyme inhibition.

Topoisomerase II and Horseradish Peroxidase Binding

To study the interaction of phenol with topoisomerase II following peroxidase-mediated activation, an experiment was carried out to measure the covalent binding of [^{14}C]phenol equivalents to the enzyme using AMS. The experimental design for the detection of radiolabeled metabolites bound to topoisomerase II is shown in Table 3. Reactions 1 and 2 were performed to determine the covalent binding of the phenol metabolites to topoisomerase II

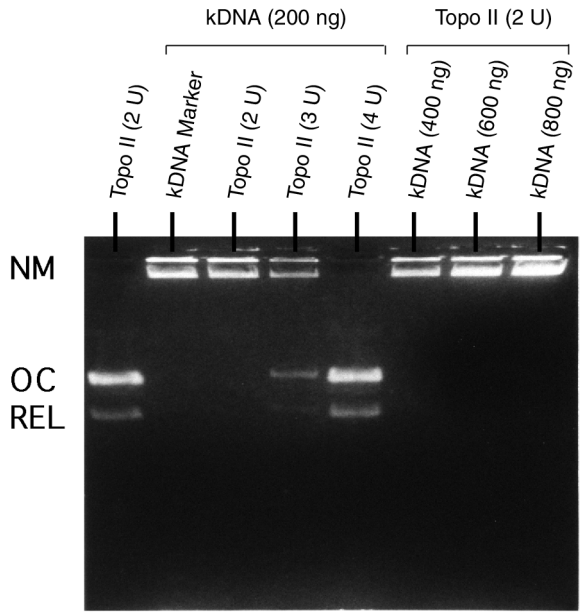

Figure 12. Effects of different concentrations of enzyme and DNA on the inhibition of topoisomerase II activity by 4,4′-biphenol following HRP-mediated bioactivation. NM: kDNA networks exhibiting no migration from the origin; OC: open circular DNA; REL: relaxed DNA. The open circular and relaxed DNA result from topoisomerase II activity allowing the smaller DNA to migrate through the gel.

and HRP, respectively. Reaction 3 was designed to investigate the possible protective effect of GSH on the binding of phenol metabolites to topoisomerase II. Reaction 4 was a background control to allow a correction for the presence of naturally occurring ^{14}C in acrylamide gel and topoisomerase II. Reaction 5 was a negative control, since 100 μM phenol in the absence of metabolic activation does not inhibit topoisomerase II and, therefore, presumably would exhibit low binding activity toward topoisomerase II.

The results of the AMS analysis for protein adduction are shown in Table 7. Following the peroxidase-mediated bioactivation of phenol, an average of 29 modern was recovered in the topoisomerase II bands on the SDS-PAGE gel. This translates to 33.4 fmol of [^{14}C]phenol equivalents bound per 1 pmol of topoisomerase II. Since 2.8% of phenol in the reaction mixture was ^{14}C-labeled phenol, the actual binding of phenol would be approximately 1.2 pmol of phenol equivalents per 1 pmol of topoisomerase II monomer. In contrast, only 0.9 fmol of [^{14}C]phenol per 1 pmol of topoisomerase II was detected in the absence of metabolic activation.

To investigate the nature and specificity of the reactive phenolic species involved in the binding of topoisomerase II, GSH was added to the reaction mix prior to the addition of topoisomerase II. The results are shown in Table 7. Following the addition of GSH, a significant decrease in [^{14}C]phenol equivalents binding to topoisomerase II was observed. Binding decreased from 33.4 (fmol/pmol enzyme) to 13.6 (fmol/pmol enzyme), an approximate 59% reduction. Combined with our previous observations that GSH protects the activity of the enzyme, these results provide evidence that the binding of a reactive metabolite to topoisomerase II leads to the inhibition of the enzyme.

The molecular weight of HRP is 44 kDa, making it easily separable from the 170 kDa topoisomerase II by SDS-PAGE. Horseradish peroxidase binding was detected at 1.1 fmol of [^{14}C]phenol equivalents per 1 pmol of HRP (Table 7). Fol-

Table 7. Adduction of [^{14}C]Phenol Equivalents to Topoisomerase II or Horseradish Peroxidase in Vitro Determined by Accelerator Mass Spectrometry

Protein	Covalent Binding[a] (fmol [^{14}C]phenol equivalent/pmol protein)
Topoisomerase II (complete reaction)[b]	33.4 ± 6.5[c]
Topoisomerase II (complete reaction plus GSH)[d]	13.6 ± 11.1[e]
Topoisomerase II (complete reaction without HRP)	0.9 ± 1.8
HRP (complete reaction)[b]	1.1 ± 0.2
HRP (complete reaction plus GSH)[d]	0.9 ± 0.2

[a] Mean ± SD of two to three experiments. Three replicates were performed for each experiment. The net radioactivity was obtained by subtracting the background ^{14}C radioactivity (due to natural ^{14}C occurrence in the polyacrylamide gel and to the ^{14}C content of phenol or its metabolites tailing through the gel) from the measured ^{14}C content of the sample.

[b] Complete reaction contained 2.8 μM [^{14}C]phenol, 97.2 μM nonradioactive phenol, topoisomerase II, and topoisomerase II assay buffer.

[c] Differs significantly from the binding detected in the complete reaction without HRP ($P \leq 0.05$; PFLSD).

[d] Reduced GSH was added after a 1-hour complete reaction containing [^{14}C]phenol, HRP, and H_2O_2 at 37°C.

[e] Differs significantly from the binding detected in the complete reaction and in the complete reaction without HRP ($P \leq 0.05$; PFLSD).

lowing the addition of GSH to the HRP reaction, no change was observed in the recovery of [^{14}C]phenol equivalents bound to HRP.

Effects of Modification of the Assay Conditions

As reported above, in the presence of a peroxidase activation system, topoisomerase II inhibition was seen at much lower concentrations for phenol, 2,2′-biphenol, and 4,4′-biphenol. The initial studies were conducted using the standard topoisomerase assay protocol recommended by the supplier of the enzyme. In addition, high peroxidase and H_2O_2 concentrations were employed for bioactivation, with incubations being performed for 1 hour to ensure that metabolism was complete, and to minimize effects due to short-lived radical species. Reduced GSH was also shown to protect the topoisomerase enzyme from inhibition by activated 4,4′-biphenol in spite of the relatively high levels of dithiothreitol in the assay buffer. This suggested that the assay conditions might significantly influence the outcome of the topoisomerase assay. Furthermore, subsequent spectrophotometric studies indicated that activation occurred very rapidly; this may have influenced the assay results for some metabolites because degradation of the inhibitory species may have occurred during the remaining 1-hour incubation.

In the next phase of experiments, the assay conditions were modified to determine the effect these modifications would have on the experimental results. The peroxidase and H_2O_2 concentrations were reduced and the incubation times were shortened to minimize degradation of the reaction products. In addition, metabolites that we had not previously tested with metabolic activation were tested for inhibitory effects following incubation with the peroxidase activation system. A series of DNA and topoisomerase titration studies were also performed using the milder peroxidase activation conditions.

Inhibition of Topoisomerase II Under Modified Conditions

Using the modified conditions, all of the benzene metabolites that were tested inhibited the ability of topoisomerase II to decatenate kDNA. The inhibitors themselves fell into two distinct classes, each of which is described below. The first group consisted of metabolites that were inhibitory when added directly to the enzyme. The second and larger group of metabolites required bioactivation by peroxidase enzymes to inhibit topoisomerase or for inhibition to be seen at low micromolar concentrations. For this second group of inhibitors, the order in which the reagents were added to the topoisomerase II assay did not substantially alter the inhibitory effects.

Direct-Acting Inhibitors

1,4-Benzoquinone, and *t,t*-muconaldehyde inhibited topoisomerase II when added directly to the enzyme prior to addition of the assay buffer and kDNA. This modified reaction assembly procedure was necessary to prevent 1,4-benzoquinone and *t,t*-muconaldehyde from reacting with components of the assay buffer. When these two compounds were mixed with the buffer prior to enzyme addition, the inhibitory effects were greatly reduced. Spectrophotometric studies (data not shown) indicated that dithiothreitol, a sulfhydryl-containing compound present in the assay buffer, could react directly with these benzene metabolites to form UV-absorbing products. The binding of the reactive metabolites to the sulfhydryl groups of dithiothreitol would protect the topoisomerase II enzyme from inhibition. Table 8 shows the metabolites and concentrations

Table 8. Benzene Metabolites That Are Direct Inhibitors of Topoisomerase II[a]

Chemical	Concentration (μM)	Inhibition[b]
1,4-Benzoquinone	500	++
	100	++
	10	+
	1	−
t,t-Muconaldehyde	100	++
	10	+/−
m-Amsacrine (positive control)	3,000	++
Ethanol (control)	3.3% (v/v)	−
Water (control)	3.3% (v/v)	−

[a] The benzene metabolites 1,4-benzoquinone and *t,t*-muconaldehyde were added directly to the topoisomerase II enzyme; the 10× assay buffer, kDNA, and water were added shortly thereafter. The reaction was initiated by placing the reaction tube into a 37°C water bath for 1 hour. Each assay was performed at least three times.

[b] Inhibition reported as: − no inhibition of enzyme activity; + partial inhibition; ++ total inhibition; +/− inconsistent inhibitory results.

tested and indicates the concentrations at which inhibitory effects were seen. 1,4-Benzoquinone inhibited topoisomerase II at concentrations at or above 10 μM. *t,t*-Muconaldehyde exhibited consistent inhibitory effects at concentrations of 100 μM and above. Inhibition of topoisomerase II was also seen at lower concentrations of *t,t*-muconaldehyde, but the effects were inconsistent.

Inhibitors Requiring Peroxidase-Mediated Bioactivation

Studies performed using the milder activation conditions indicated that phenol, 4,4′-biphenol, 2,2′-biphenol, hydroquinone, catechol, and 1,2,4-benzenetriol all required bioactivation by peroxidase in the presence of H_2O_2 to inhibit topoisomerase II at low micromolar concentrations (Table 9). Direct addition of the metabolic products to the topoisomerase did not substantially alter the inhibitory concentrations. 2,2′-Biphenol was the only compound from this group to give somewhat variable results. Activated 2,2′-biphenol consistently inhibited topoisomerase II at concentrations of 100 μM or greater. Inconsistent results were seen at the lower 10 μM concentration, however.

Table 9. Benzene Metabolites That Require Peroxidase Activation to Inhibit Topoisomerase II[a]

Chemical	Concentration (μM)	Inhibition[b]
Phenol	100	++
	10	++
	1	−
4,4′-Biphenol	100	++
	10	++
	1	−
2-2′-Biphenol	100	++
	10	+/−
Hydroquinone	100	++
	10	++
	1	+
	0.1	−
Catechol	100	++
	10	++
	1	−
1,2,4-Benzenetriol	100	++
	10	++
	1	−
m-Amsacrine (positive control)	3,000	++
Controls[c]		−

[a] All the compounds in this table with the exception of 2,2′-biphenol were incubated with 0.07 U/mL HRP and 55 μM H_2O_2 for 5 min, followed by the addition of the 10× assay buffer, kDNA, and topoisomerase II enzyme. 2,2′-Biphenol was incubated with 0.1 U/mL HRP and 55 μM H_2O_2; all other steps were the same.

[b] Inhibition reported as: − no inhibition of enzyme activity; + partial inhibition; ++ total inhibition; +/− inconsistent inhibitory results.

[c] Control exposures were to HRP (0.07–0.1 U/mL), H_2O_2 (55 μM), DMSO (0.03–3.3%), or water.

DNA Titration Experiments Using Reduced Oxidizing Conditions

Using the milder activation conditions, DNA titration experiments with phenol were performed using 100 μM peroxidase-activated phenol, 4 U of topoisomerase II, and from 200 to 2,000 ng of kDNA per incubation. Topoisomerase II activity was completely inhibited at all kDNA concentrations but the highest 2,000-ng level, which showed a partial protection of enzyme activity (Table 10). Similar experiments were performed with the phenol metabolites 4,4′-biphenol and 2,2′-biphenol. DNA titration experiments with 100 mM peroxidase-activated 2,2′-biphenol resulted in a partial return of enzyme activity at 1,000 and 2,000 ng of added kDNA (Table 10) similar to the enzyme activity seen with phenol. In contrast, DNA titration studies in presence of 100 μM peroxidase-activated 4,4′-biphenol yielded no restoration of enzyme activity

Table 10. Recovery of Topoisomerase II Activity in Phenolic Metabolites of Benzene After Peroxide-Mediated Activation and Supplementation with Various Amounts of kDNA or Topoisomerase II Enzyme[a]

	Phenol	2,2′-Biphenol	4,4′-Biphenol
kDNA (ng/reaction)			
DMSO (control)	++	++	++
200	−	−	−
300		−	
400	−	−	−
500		−	
600			−
700	−		
800			−
1,000	−	+	−
2,000	+	+	−
Topoisomerase II Enzyme (units)			
DMSO (control)	++	++	++
4			−
6	−	−	+
8	−	−	++
10	−	−	
12	−	−	
14	−	−	
16	−	−	

[a] A − indicates no topoisomerase II decatenation activity; + indicates partial activity; and ++ indicates complete activity. A blank cell indicates that the level of enzyme activity for that metabolite was not assayed.

when 200 to 2,000 ng of kDNA were added to the incubation (Table 10). These titration assays demonstrated that an increase in the amount of kDNA in the assay mixture allows for partial recovery of topoisomerase II activity from the inhibitory effects of 100 μM bioactivated phenol or 2,2′-biphenol, whereas no protection of enzyme activity was seen for 100 μM bioactivated 4,4′-biphenol. These results indicate that under metabolic conditions allowing the formation of 2,2′-biphenol and its oxidative products from phenol, inhibition of topoisomerase II is likely to be due to a DNA-interactive mechanism.

Topoisomerase II Enzyme Titration Experiments

Employing the less extensive activation conditions, enzyme titrations were conducted with phenol and 2,2′-biphenol, and with 4,4′-biphenol using 100 μM peroxidase-activated compound, 400 ng of kDNA, and between 4 and 16 U of topoisomerase II. No restoration in topoisomerase II activity, as measured by kDNA decatenation, was observed over the range of enzyme concentrations tested for bioactivated phenol and 2,2′-biphenol (Table 10). Similar to that seen using the stronger peroxidase conditions, increasing the amount of topoisomerase II in the incubations of bioactivated 4,4′-biphenol resulted in a partial recovery of topoisomerase II decatenating activity at 6 U of enzyme and a complete recovery of activity with 8 U of topoisomerase II enzyme (Table 10).

These results demonstrate that inhibition of topoisomerase II by bioactivated 4,4′-biphenol is the result of a direct effect of the inhibitor on the enzyme. It should also be noted that no more than 16 U of topoisomerase II were used in the enzyme titration experiments, because levels of enzyme greater than or equal to 25% of total reaction volume (16 U or 8 μL) are capable of causing protein-induced inhibition of the topoisomerase II enzyme (TopoGEN 1994). Previously, Subrahmanyam and O'Brien (1985a,b) showed that the peroxidative activation of 4,4′-biphenol produces primarily diphenoquinone and that this metabolite is capable of binding to protein. In addition, the results presented above have demonstrated that peroxidase-activated 4,4′-biphenol is capable of inhibiting topoisomerase II activity. To demonstrate directly that diphenoquinone was capable of inhibiting human topoisomerase II, diphenoquinone was synthesized and tested for inhibitory effects on topoisomerase II in the in vitro assay.

Inhibition of Topoisomerase II Activity by Diphenoquinone, the Primary Oxidative Metabolite of 4,4′-Biphenol

Diphenoquinone completely inhibited topoisomerase II at a concentration of 100 μM and partially inhibited enzyme activity at a concentration of 10 μM (Table 11) when directly added to the enzyme. These results are similar to those seen above with 1,4-benzoquinone and *t,t*-muconaldehyde, both directly reactive metabolites. When diphenoquinone was added to the assay buffer prior to the addition of topoisomerase II, it was able to inhibit the enzyme only at substantially higher concentrations. This is likely because of the reactive nature of diphenoquinone which, when added to a reaction buffer containing serum proteins and dithiothreitol, can react with available proteins and thiols, rendering it unavailable for interactions with topoisomerase II.

Table 11. Diphenoquinone Inhibition of Human Topoisomerase II[a]

Chemical	Concentration (μM)	Inhibition[b]
Diphenoquinone	100	++
	10	+
	1	+/−
	0.1	−
m-Amsacrine (positive control)	3,000	++
DMSO (solvent control)	3.3%	−

[a] Each assay was performed at least three times.

[b] Inhibition reported as: − no inhibition of enzyme activity; + partial inhibition; ++ total inhibition; +/− inconsistent inhibitory results.

These results showed that a number of the known and putative benzene metabolites could inhibit topoisomerase II when converted to reactive or bioactive intermediates. The next objective of these studies was to determine which if any of these metabolites would be capable of inhibiting topoisomerase II in a cell culture system. HL-60 cells were selected for this series of studies because they are bone marrow–derived cells of human origin that contain considerable peroxidase activity.

Dose-Response Studies of Benzene Metabolites in Human HL-60 Cells

To identify conditions under which topoisomerase II inhibition was likely to occur, an extensive series of cytotoxicity studies was performed using HL-60 cells over a range of metabolite concentrations, H_2O_2 concentrations, and sampling times (Appendix A). HL-60 cells were exposed to the benzene metabolites 1,2,4-benzenetriol, 2,2′-biphenol, 4,4′-biphenol, catechol, hydroquinone, and phenol over a concentration range of 0 to 1,000 μM with either 0, 1, 10, or 20 μM H_2O_2. Cells were sampled at five time points over a 48-hour period. Hydrogen peroxide was

used to increase the peroxidase metabolism of the HL-60 cells. The results of the cell viability experiments were examined to identify concentrations and exposure times that were not significantly different from controls at a point prior to a rapid onset of extensive (nearly 100%) cytotoxicity. For most metabolites, the cytotoxic effects depended on concentration and time. The phenol-exposed cells, however, did not demonstrate cytotoxicity at any of the concentrations tested (Appendix A, Table A.1). Cell viability in 2,2′-biphenol–exposed cells was significantly decreased at 500 and 1,000 μM (Appendix A, Table A.2). The 500 μM exposure resulted in less than a 40% decrease in the number of viable cells, however, and therefore was not further tested for inhibition of topoisomerase II. The remaining chemicals tested—4,4′-biphenol, 1,2,4-benzenetriol, catechol, and hydroquinone—all showed significant rapid decreases in cell viability at concentrations less than or equal to 500 μM (Appendix A, Tables A.3 through A.6). The lowest chemical and H_2O_2 concentration that produced a rapid and extensive decrease in cell viability was selected for testing at the time point prior to the onset of significant cytotoxicity.

Inhibition of Topoisomerase II in Treated Human HL-60 Cells

Using the concentrations and conditions identified in the cell viability experiments, catechol, hydroquinone, 1,2,4-benzenetriol, and 4,4′-biphenol were tested for their ability to inhibit topoisomerase II immediately prior to the onset of toxicity. The putative benzene metabolite 4,4′-biphenol was initially used as a model compound to determine whether benzene metabolites were capable of inhibiting topoisomerase II enzyme activity in vitro. As indicated above, peroxidase-activated 4,4′-biphenol and its product diphenoquinone have both been shown to be potent inhibitors of human topoisomerase II in vitro. Based on the cell viability studies, the 500-μM 4,4′-biphenol concentration in the presence of 10 μM H_2O_2 and the 8-hour time point were selected for testing. The concentration and time responses for this concentration of 4,4′-biphenol is shown in Figure 13. At the 8-hour time point, the percentage of viable cells was similar in the control and 4,4′-biphenol–treated cultures (100% and 87%, respectively) but decreased rapidly to 14% and 0% at the 24-hour and 48-hour time points. At the 8-hour time point, a strong and significant decrease in the topoisomerase II enzyme activity of the 4,4′-biphenol + H_2O_2–treated cells was seen as compared with an equal number of viable control cells (one-group t test, $P \leq 0.05$). A representative gel showing the amounts of decatenated kDNA across several dilutions of the nuclear extract is shown in Figure 14. Representative area-under-the-curve (AUC) measurements obtained using image analysis are also presented in Figure 14. By comparing the corresponding nuclear extract dilutions from the treated and control cultures, the amount of topoisomerase II activity in the cells can be estimated. As can be seen in this and the following figures, there is variability in response among the dilutions and, in some cases, the more dilute extracts show higher activity than the less dilute extracts. This phenomenon has been seen by others working in this field (F Gieseler, personal communication, 1999) and is believed to be due to complex interactions between topoisomerase II, interacting proteins such as histones, 14-3-3-proteins, and GADD45, as well as residual DNA, salts, and cofactors. By comparing the activity in the treated and control cells across a series of dilutions, the relative level of topoisomerase activity in the exposed cells can be estimated. The topoisomerase II activity of the 4,4′-biphenol–treated cellular extract averaged across the dilutions and four separate experiments was 52% of that of the controls (Table 12).

Similar studies were performed using the benzene metabolites hydroquinone, 1,2,4-benzenetriol, and catechol. For hydroquinone, the 50-μM concentration in the presence of 10 μM H_2O_2 at the 2-hour time point was selected for testing. Cell viability at this concentration and time was 98%, which decreased to 44% by 4 hours and 6% by 8 hours (Figure 13). The topoisomerase II activity of cells harvested at the 2-hour time point averaged 45% of the controls, a decrease that was statistically significant (one-group t test, $P \leq 0.05$; Table 12). A representative gel and image measurements are shown in Figure 15.

For the 1,2,4-benzenetriol–treated cells, the 100-μM concentration without H_2O_2 at the 2-hour time point was selected. Cell viability was 90% at the 2-hour time point, and decreased to 59% and 51% by 4 hours and 8 hours, respectively (Figure 13). The topoisomerase II activity of the nuclear extracts obtained at the 2-hour time point was significantly decreased, averaging 48% of the control values (one-group t test, $P \leq 0.05$; Table 12). Representative gel and image measurements are shown in Figure 16.

For the catechol-treated cells, the 500-μM catechol and 10-μM H_2O_2 concentrations at the 2-hour time point were selected for assay. Under these conditions, cell viability decreased from 97% at the 2-hour time point to 64% and 6% at the 8-hour and 24-hour time points (Figure 13). No decrease in topoisomerase II activity was seen in the catechol-treated cells (Table 12). Representative gel and image measurements are shown in Figure 17.

Consistent with the results in the previous viability experiments, the viability of the hydroquinone-exposed, 1,2,4-benzenetriol–exposed, and catechol-exposed cells at the selected time points did not differ significantly from

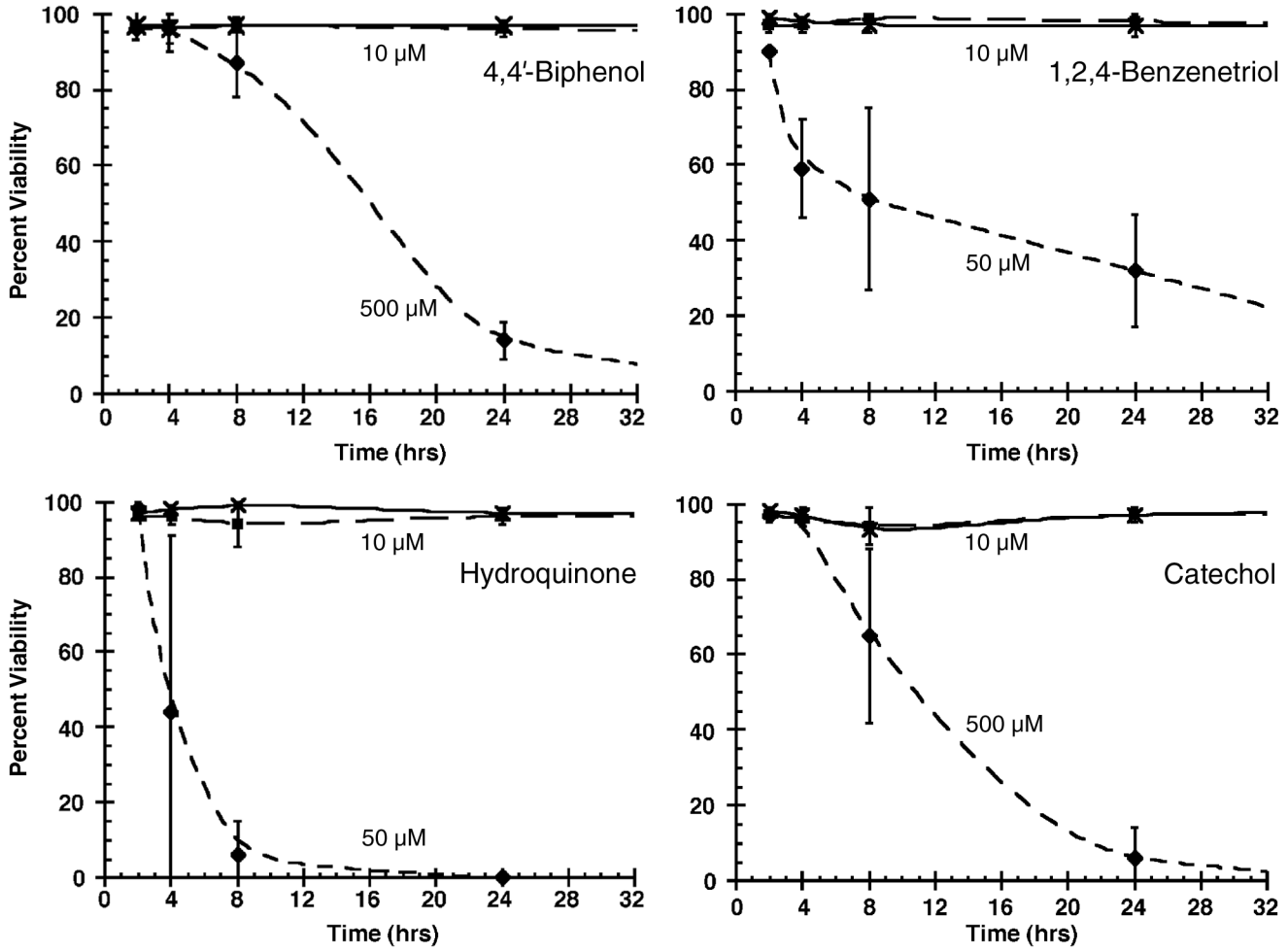

Figure 13. Cytotoxicity seen in human HL-60 cells exposed to two concentrations each of 4,4′-biphenol, hydroquinone, 1,2,4-benzenetriol, or catechol. Percent viability of the exposed cells is plotted over time. Cells were exposed to 0.1% DMSO or the indicated concentrations of each chemical in the presence of 10 μM H_2O_2, with the exception of 1,2,4-benzenetriol with which no H_2O_2 was included. In each panel, ×—× indicates the DMSO exposure. See Appendix A for these and other data.

the control HL-60 cells (Table 13). The viability of cells treated with 4,4′-biphenol, however, was slightly but significantly decreased compared with that in the control cells (*t* test, $P \leq 0.05$). For the topoisomerase II assays in HL-60 cells and subsequent studies with mouse bone marrow cells, all extractions were performed using an equal number of viable cells to correct for any differences whether the cell viability results differed statistically or not.

Table 12. Inhibition of Topoisomerase II in the Human HL-60 Cell Line by Known and Putative Benzene Metabolites

Test Chemical	Exposure Concentration	Exposure Time (hours)	*n* Experiments	Topoisomerase II Activity[a]
Hydroquinone	50 μM + 10 μM H_2O_2	2	3	44.9 ± 20.1[b]
1,2,4-Benzenetriol	100 μM	2	3	47.8 ± 19.6[b]
Catechol	500 μM + 10 μM H_2O_2	2	3	97.3 ± 20.6
4,4′-Biphenol	500 μM + 10 μM H_2O_2	8	4	52.3 ± 12.6[b]

[a] Data are expressed as percentages of control topoisomerase II activity and presented as means ± SD.

[b] Significantly different from controls ($P \leq 0.05$; one-group *t* test).

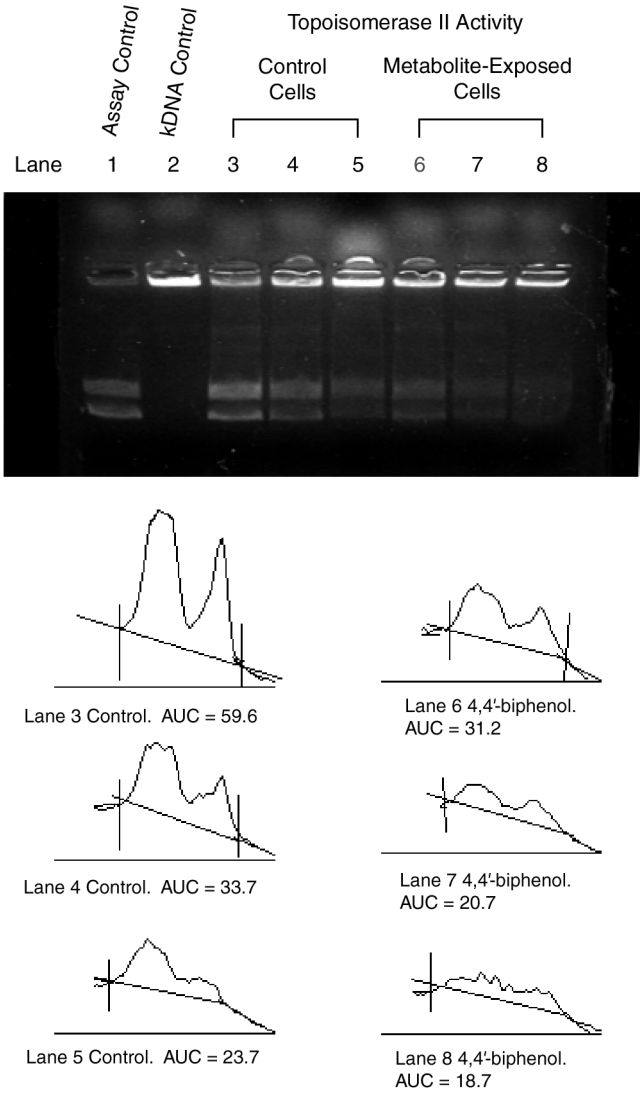

Figure 14. (Top) Topoisomerase II enzyme assay of human HL-60 control cells and cells exposed to 500 μM 4,4′-biphenol + 10 μM H_2O_2. (Bottom) Representative AUC measurements of decatenated kDNA used to determine the topoisomerase II activity in control and exposed cells. Lanes 3 and 6: 1:10 dilution of the nuclear extract; lanes 4 and 7: 1:5 dilution; lanes 5 and 8: no dilution.

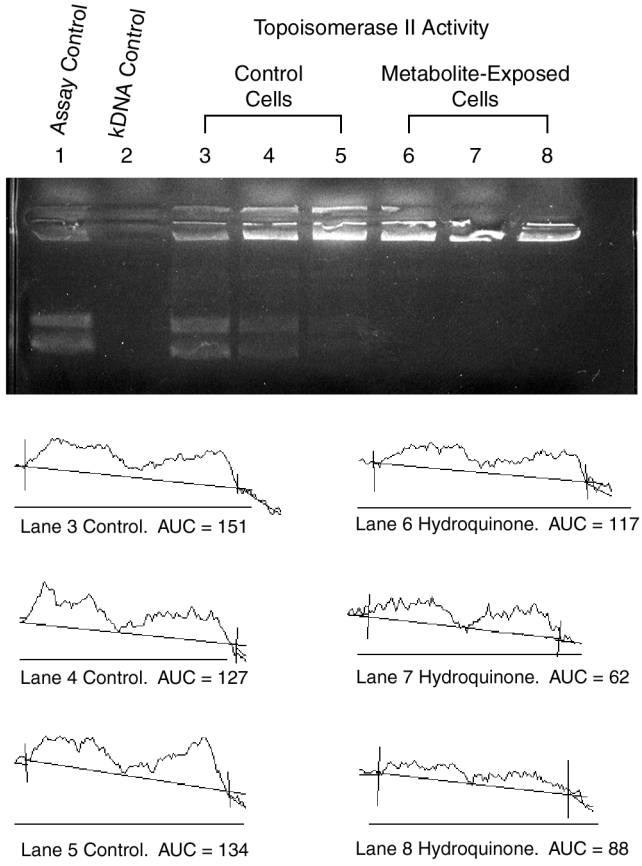

Figure 15. (Top) Topoisomerase II enzyme assay of human HL-60 control cells and cells exposed to 50 μM hydroquinone + 10 μM H_2O_2. (Bottom) Representative AUC measurements of decatenated kDNA used to determine the topoisomerase II activity in control and exposed cells. Lanes 3 and 6: 1:20 dilution of the nuclear extract; lanes 4 and 7: 1:10 dilution; lanes 5 and 8: 1:5 dilution.

Having demonstrated that a human promyelocytic leukemic cell line treated with several of the benzene metabolites exhibited significant decreases in topoisomerase II activity during the period immediately preceding cell toxicity in vitro, we next performed similar assays in the

Table 13. Viability of Human HL-60 Cells Exposed to Benzene Metabolites[a]

Test Chemical	Exposure Concentration	*n* Experiments	Control Cells	Exposed Cells
Hydroquinone	50 μM + 10 μM H_2O_2	3	94.7 ± 0.6	93.0 ± 2.6
1,2,4-Benzenetriol	100 μM	3	95.3 ± 0.6	95.3 ± 3.1
Catechol	500 μM + 10 μM H_2O_2	3	95.3 ± 0.6	93.0 ± 2.6
4,4′-Biphenol	500 μM + 10 μM H_2O_2	4	93.3 ± 3.3	86.8 ± 5.1[b]

[a] Data are expressed as percentages of viable cells at the selected time point and presented as means ± SD.

[b] Significantly different from controls ($P \leq 0.05$; *t* test).

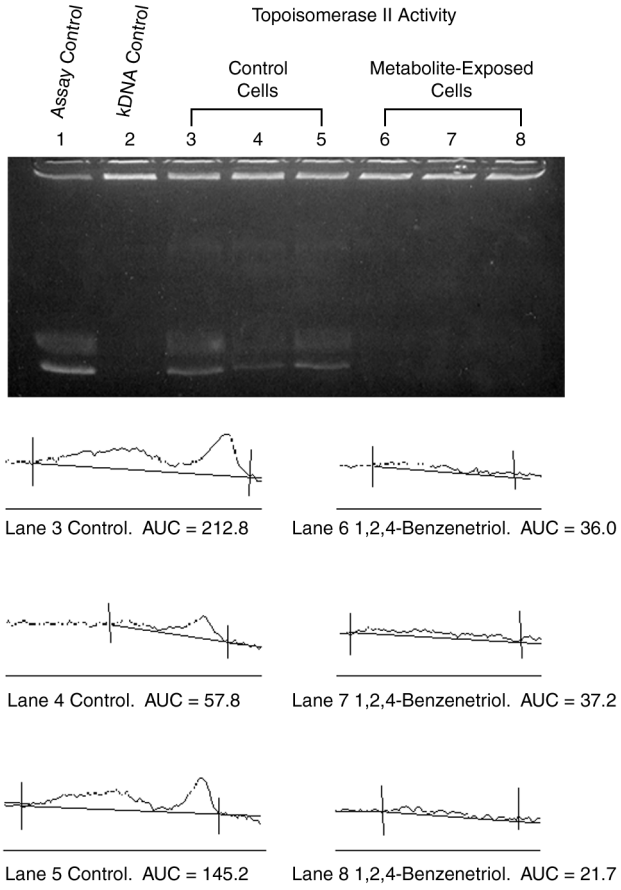

Figure 16. (Top) Topoisomerase II enzyme assay of human HL-60 control cells and cells exposed to 100 μM 1,2,4-benzenetriol. (Bottom) Representative AUC measurements of decatenated kDNA used to determine the topoisomerase II activity in control and exposed cells. Lanes 3 and 6: 1:20 dilution of the nuclear extract; lanes 4 and 7: 1:10 dilution; lanes 5 and 8: 1:5 dilution.

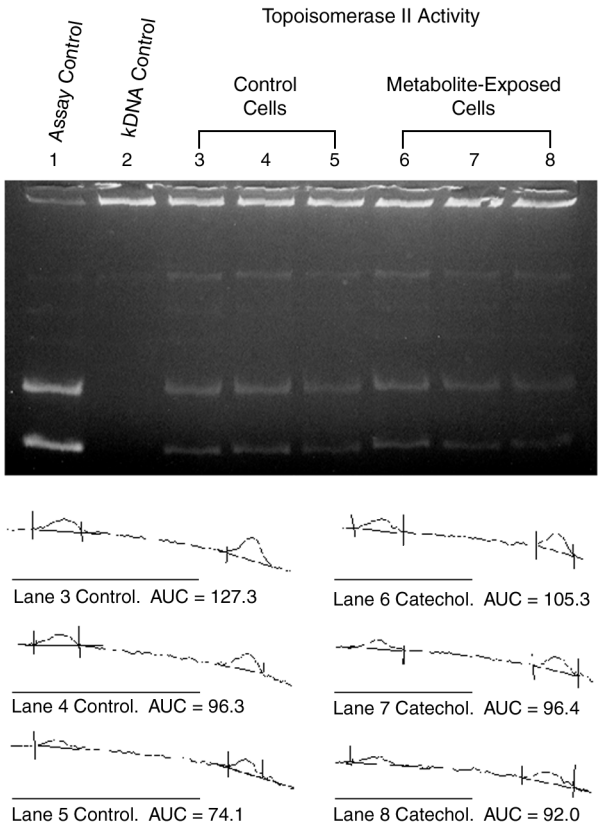

Figure 17. (Top) Topoisomerase II enzyme assay of human HL-60 control cells and cells exposed to 500 μM catechol + 10 μM H_2O_2. (Bottom) Representative AUC measurements of decatenated kDNA used to determine the topoisomerase II activity in control and exposed cells. Lanes 3 and 6: 1:5 dilution of the nuclear extract; lanes 4 and 7: 1:10 dilution; lanes 5 and 8: 1:20 dilution.

nuclear extracts of mouse bone marrow cells following administration of benzene in vivo.

Inhibition of Topoisomerase II in Vivo

Using the work of Bodell and associates (1993) and Chen and colleagues (1994a) as a starting point, pilot studies were conducted to determine the dose of benzene that could be administered to 6-week-old male B6C3F_1 mice without producing overt cytotoxic effects in the nucleated cells of the bone marrow. Preliminary studies indicated a dose of 440 mg/kg benzene could be given for 3 consecutive days to the mice without producing a decrease in the recovery of viable bone marrow cells. Although cytotoxicity was not seen in these initial studies, previous studies from our laboratory have shown that a single oral dose of 440 mg/kg is capable of inducing chromosomal damage in mouse bone marrow cells (Chen et al 1994a).

For the topoisomerase II inhibition studies, male B6C3F_1 mice were given either corn oil or 440 mg/kg benzene in corn oil by oral gavage for 3 days. Twenty-four hours later the mice were killed and the activity of topoisomerase II in the nuclear protein extracts of the femoral bone marrow cells was determined by the kDNA decatenation assay. The amount of decatenated kDNA was quantified by image analysis and expressed as a percentage of the topoisomerase II enzyme activity measured in control mice. Representative gel and AUC measurements obtained using image analysis are presented in Figure 18. The topoisomerase II activity in the bone marrow cells of the benzene-exposed mice was found to be an average of 61% of that of the control mice, a level significantly different from that seen in the control animals (one-group t test; $P \leq 0.05$) (Table 14). The cell viability of control and benzene-exposed nucleated bone marrow cells was determined immediately prior to the extraction of the nuclear proteins, and no difference in viability was seen between the two groups (t test, $P \leq 0.05$) (Table 15).

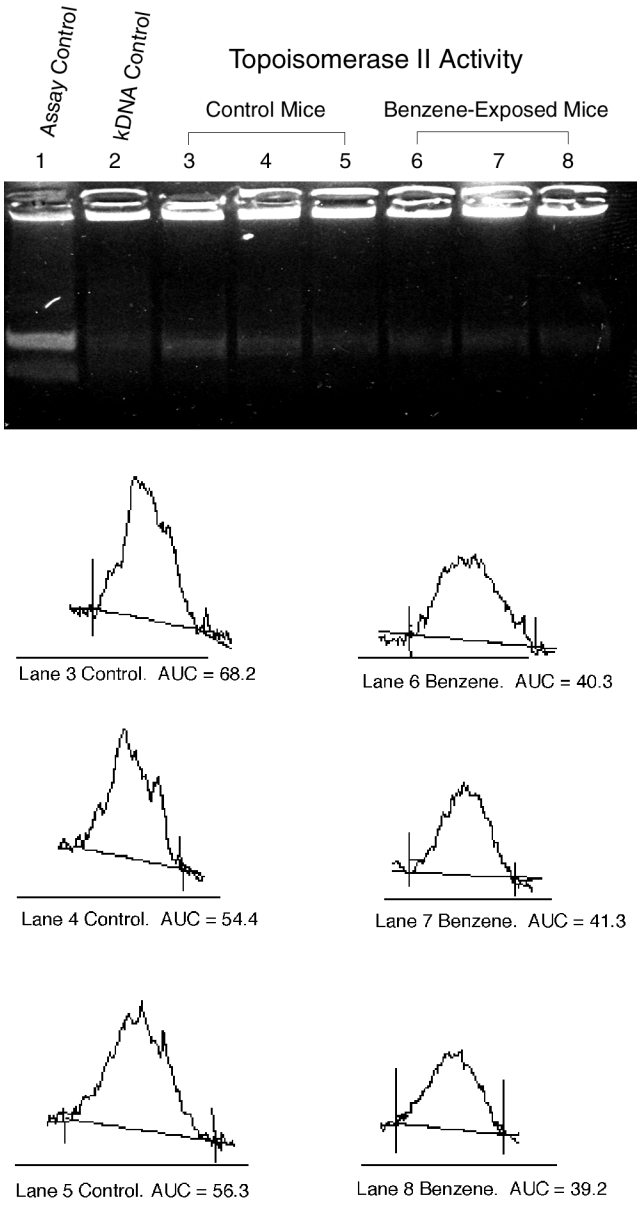

Figure 18. (Top) Topoisomerase II enzyme assay of control and benzene-exposed B6C3F$_1$ mice. Mice were exposed to either corn oil or 440 mg/kg benzene once per day for 3 days by oral gavage and killed 24 hours after the last dose. **(Bottom) Representative AUC measurements of decatenated kDNA used to determine the topoisomerase II activity in control and exposed mice.** The presence of only one peak indicates lower amounts of total topoisomerase II obtained from mouse bone marrow cells compared with the HL-60 cells shown in Figures 14 through 17. Lanes 3 and 6: 1:20 dilution of the nuclear extract; lanes 4 and 7: 1:10 dilution; lanes 5 and 8: 1:5 dilution.

Topoisomerase II Protein Levels and [^{14}C]Benzene in Vivo Binding Studies

We were unable to detect the 170-kDa topoisomerase II monomer protein in either control or benzene-exposed mice either visually or using our image scanning method.

Table 14. Inhibition of Topoisomerase II in the Nucleated Bone Marrow Cells of Male B6C3F$_1$ Mice Exposed to Benzene by Oral Gavage

Experiment	Enzyme Activity in Exposed Cells[a]
1	54.3 ± 5.1
2	68.3 ± 10.0
3	59.3 ± 16.6
Mean of all experiments	60.7 ± 11.7[b]

[a] Data are expressed as percentages of control cells and presented as means ± SD.

[b] Differs significantly from controls ($P \leq 0.05$; one-group t test).

Table 15. Viability of Nucleated Mouse Bone Marrow Cells Unexposed or Exposed in Vivo to Benzene[a]

Experiment	Control Cells	Exposed Cells
1	71	70
2	71	70
3	80	79
Mean ± SD	74.0 ± 5.2	73.0 ± 5.2

[a] Values are expressed as percentages of viable cells at the selected time point.

This is probably because of the small numbers of nucleated cells in the mouse femurs; we were able to recover only between 5×10^6 and 1×10^7 total nucleated bone marrow cells in the mouse experiments. The amount of topoisomerase II extractable from this number of cells provided sufficient enzyme to measure enzyme activity, but there was not enough protein to detect by ECL-plus Western blotting detection.

Using AMS combined with molecular weight standards, we were able to detect protein binding to the transfer membrane and estimate the weights of the proteins to which [^{14}C]benzene was bound. No increase in binding was detected at the membrane section at the molecular weight corresponding to the 170-kDa topoisomerase II monomer. The lack of visible protein made interpretation of the radiolabeled benzene binding results difficult. There were indications that [^{14}C]benzene did bind to proteins between 160 kDa and 127 kDa. These results were somewhat inconsistent, however, and should be considered as preliminary. Due to the small numbers of cells available in the mouse femur and our inability to confirm the recovery of mouse topoisomerase II on the membrane, additional mechanistic investigations were conducted using HL-60 cells.

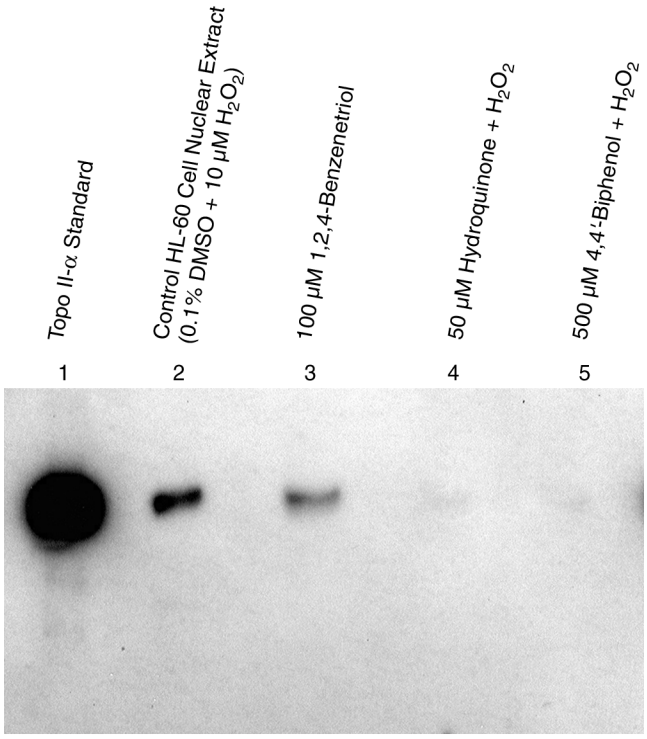

Figure 19. Topoisomerase II protein levels in nuclear extracts from human HL-60 cells unexposed or exposed to benzene metabolites. Equal amounts of total nuclear extract protein were separated by SDS-PAGE, transferred onto PVDF membrane, and topoisomerase II-α protein levels were detected by ECL-plus. A representative Western blot from 1 of 3 experiments is shown.

Topoisomerase II Protein Levels and [^{14}C]Benzene Metabolite Binding Studies in Human HL-60 Cells

To understand the mechanism of benzene metabolite inhibition of topoisomerase II, topoisomerase II protein levels and benzene metabolite binding studies were performed in the human HL-60 cell line using the same exposure conditions that resulted in inhibition of topoisomerase II activity. Under these experimental conditions, the HL-60 cells exposed to hydroquinone, 1,2,4-benzenetriol, and 4,4′-biphenol were all shown to have significantly decreased levels of the 170-kDa topoisomerase II monomer compared with levels in control HL-60 cells (Figure 19). In these same experiments, we were unable to detect any significant PVDF membrane–bound ^{14}C within the 170-kDa region by liquid scintillation counting.

DISCUSSION

The results of these studies demonstrate that benzene is capable of inhibiting topoisomerase II in the nucleated bone marrow cells of mice and that the benzene metabolites hydroquinone, 1,2,4-benzenetriol, and the putative metabolite 4,4′-biphenol are capable of inhibiting topoisomerase II activity in the human HL-60 cell line. Additional studies using isolated human topoisomerase II showed that, when converted to bioactive species, most of benzene's unconjugated metabolites are capable of inhibiting isolated topoisomerase II in vitro at relatively low concentrations. Similar inhibitory effects of hydroquinone and 1,4-benzoquinone on topoisomerase II in vitro have been reported (Hutt and Kalf 1996). As described above, DNA titration and binding studies using isolated topoisomerase II in vitro indicated that topoisomerase inhibition by benzene metabolites potentially can occur through both protein- and DNA-interactive mechanisms. Bioactivated 4,4′-biphenol inhibited topoisomerase II through a direct interaction with the enzyme, whereas inhibition by bioactivated 2,2′-biphenol occurred through a DNA interaction. Further mechanistic studies in HL-60 cells showed that hydroquinone, 1,2,4-benzenetriol, and 4,4′-biphenol caused a decrease in the protein levels of topoisomerase II in this leukemia cell line. These results provide the first direct evidence that benzene is capable of inhibiting enzymatic activity of topoisomerase II in vivo and in cells in culture. Furthermore, the studies in HL-60 cells indicate that the bioactive metabolites hydroquinone, 1,2,4-benzenetriol, and possibly the putative metabolite 4,4′-biphenol are likely candidates for the benzene metabolites that inhibit topoisomerase II in vivo.

In our initial studies, a number of benzene metabolites were screened by our laboratory for their inhibitory effects on human topoisomerase I and II in vitro. In these experiments, assay conditions recommended by the supplier of the enzyme were used as well as relatively long (1 hour) bioactivation incubations with high levels of peroxidase enzymes. In the later studies, shorter bioactivation periods (5 min) with reduced enzyme concentrations were selected. The results of the two series of experiments are quite consistent but illustrate how modifications in the assay conditions can significantly alter the test results. For example, by adding 1,4-benzoquinone directly to the enzyme, inhibition was seen at a 10-μM (final) concentration, whereas inhibition was seen only at 500 μM when this reactive metabolite was added to the dithiothreitol- and BSA-containing buffer. For the phenolic metabolites, incubation in the presence of a peroxidase-H_2O_2 activation system was necessary for inhibition of topoisomerase II to be seen at low micromolar concentrations. By altering the bioactivation conditions, inhibition of topoisomerase II was seen at lower concentrations for phenol, 2,2′-biphenol, and 4,4′-biphenol. A proposed pathway for the inhibition of topoisomerase II by these peroxidase-activated metabolites is shown in Figure 20. The rationale

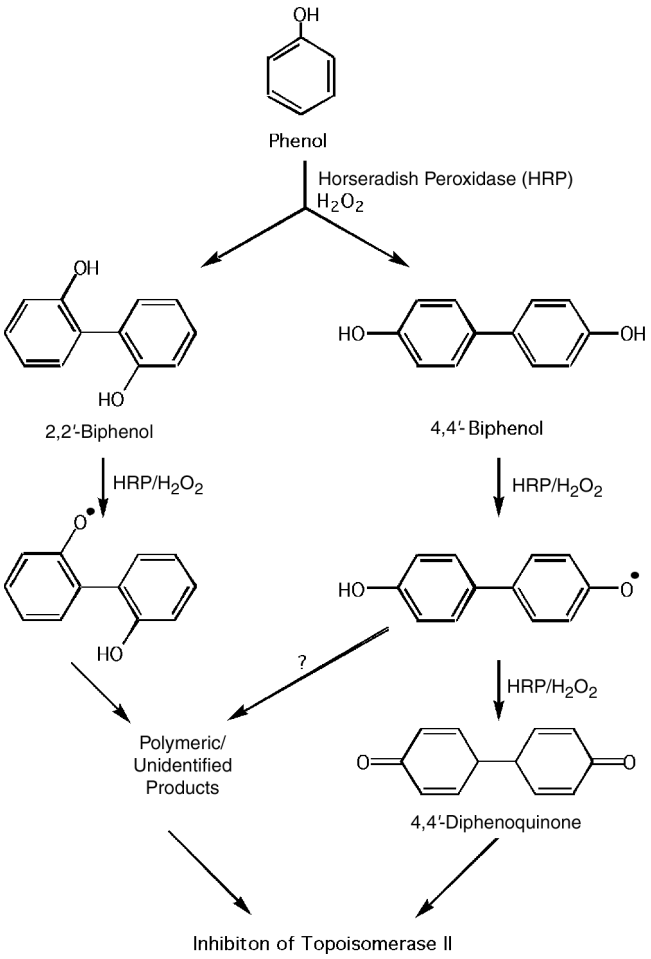

Figure 20. Proposed pathway for inhibition of human topoisomerase II during the peroxide-mediated metabolism of phenol. Note that the semiquinone radicals shown can rearrange to form carbon-centered radical species that may result in polymeric products.

for the use of a peroxidase activation system is that the bone marrow contains high levels of myeloperoxidase as well as other peroxidase enzymes (Schattenberg et al 1994; Twerdok et al 1992; Twerdok and Trush 1988), and increases in reactive oxygen species have been shown to occur in this organ following benzene administration (Kolachana et al 1993; Subrahmanyam et al 1991). In addition, relatively high levels of phenolic metabolites have been recovered in the bone marrow following benzene exposure (Rickert et al 1981).

Although the majority of the compounds assayed in the modified in vitro studies were inhibitory at the 10-μM concentration, two of the compounds, *t,t*-muconaldehyde and 2,2′-biphenol, required higher concentrations (100 μM) to inhibit the topoisomerase enzyme consistently. These compounds were sometimes observed to inhibit the enzyme at lower concentrations, however. We believe that these somewhat inconsistent results were due to instability of *t,t*-muconaldehyde in aqueous solutions and the incomplete metabolism and formation of polymers in incubations containing 2,2′-biphenol. Attempts to use higher concentrations of peroxidase and H_2O_2 to complete the metabolism of 2,2′-biphenol were problematic in that occasionally inhibition of topoisomerase II was observed in the absence of added inhibitors, making interpretation of the experiments difficult. At the concentrations of 0.07 to 0.1 U/mL HRP and 55 μM H_2O_2 used for bioactivation, however, this problem was not seen. As a result of the reactive nature and instability of certain metabolites, the probable presence of nucleophilic constituents in the topoisomerase-containing solution, and the influence of the assay conditions, we believe that the absolute inhibitory concentrations observed in the topoisomerase II assay should be interpreted with caution. Indeed, one lesson from these studies is that the standard assay conditions used to screen pharmaceutical and toxicological agents for topoisomerase-inhibitory activity may not detect certain classes of inhibitory compounds, such as those that may react with dithiothreitol or other components of the topoisomerase assay. In spite of the limitations inherent in the in vitro assay, it is still clear that most of the known and putative benzene metabolites tested inhibited topoisomerase II in vitro in the range of low micromolar concentrations when assayed under appropriate test conditions or with metabolic activation.

The DNA-interactive effects of peroxidase-activated phenol and 2,2′-biphenol are in agreement with the previous in vitro studies done by Subrahmanyam and O'Brien (1985a), who demonstrated that approximately 65% of peroxidase-metabolized [^{14}C]phenol was recovered bound to calf thymus DNA. These authors also demonstrated that, of the two primary phenol peroxidase metabolites, only 2,2′-biphenol, which continues to undergo oxidation and form polymeric products, binds to DNA (Subrahmanyam and O'Brien 1985a,b).

The results of the in vitro titration studies also show that peroxidase-activated 2,2′-biphenol does not appear to be protein-interactive. Peroxidase-activated phenol was also shown not to be highly protein-interactive under the modified conditions used. In the case of phenol, one might expect some protein interactions, since the primary peroxidase metabolites of phenol are 2,2′-biphenol, which continues to be oxidized forming DNA-interactive polymers, and 4,4′-biphenol, which is oxidized into the protein-interactive compound diphenoquinone (Subrahmanyam and O'Brien 1985a,b). The lack of protein interaction with peroxidase-activated phenol is most likely to be explained

by the in vitro peroxidase-activating conditions used for these assays. Spectrophotometric investigations under the peroxidase metabolic conditions used to bioactivate phenol in the titration studies showed a significantly lower formation of diphenoquinone from phenol as compared with incubations conducted using the strong peroxidase-activating conditions used in the initial experiments. Nonetheless, the results presented here show that peroxidase-activated phenol through the 4,4′-biphenol pathway is capable of inhibiting topoisomerase II via a protein-interactive mechanism and is consistent with the observed binding of bioactivated phenol to topoisomerase II.

Topoisomerase II inhibitors have been shown to function through a variety of mechanisms, including both protein- and DNA-interactive mechanisms (Osheroff et al 1994). One method by which DNA-interactive topoisomerase II inhibitors function is through intercalation of DNA and the stabilization of the DNA topoisomerase II enzyme complex, forming what is termed the "cleavable complex." This effectively renders the enzyme nonfunctional and ultimately results in DNA breaks. It is also possible that a DNA-interactive agent could prevent the enzyme from acting on DNA by binding at topoisomerase II recognition sites or through binding DNA in a quantity sufficient to prevent enzymatic activity (Insaf et al 1996). In either case, it should be possible to add enough DNA to the reaction to serve as a substrate for the intact topoisomerase II and allow decatenation of kDNA to occur. Even increasing the amount of kDNA added to the reaction mix by a factor of 10 did not result in a complete restoration of enzyme activity as measured by the amount of decatenated kDNA. This suggests that the inhibition may be occurring through a mechanism involving chemical interactions with both DNA and topoisomerase II. It is not possible, however, to discount that the total amount of kDNA added was insufficient to overcome the inhibitory effects completely. Since increasing topoisomerase II protein levels in conjunction with an inhibitory concentration of bioactivated 2,2′-biphenol failed to restore topoisomerase II enzyme activity, we were able to conclude that the peroxidative metabolites of 2,2′-biphenol inhibit topoisomerase II via a DNA-interactive mechanism. At this point, it is not clear whether 2,2′-biphenol inhibits binding, traps the enzyme on the DNA in a "cleavable complex," or inhibits through another mechanism. The results seen here are consistent with those reported for other topoisomerase II inhibitors, however. For example, several known topoisomerase II–inhibiting drugs—such as the anthracyclines, naphthoquinones, and other related *para*-quinone and *ortho*-quinone chemotherapeutics, which exhibit some structural similarities to the likely polymeric products formed in the oxidation of 2,2′-biphenol—have been reported to inhibit topoisomerase II through intercalation and stabilization of the cleavage complex (Frydman et al 1997; Insaf et al 1996).

In contrast to the 2,2′-biphenol mechanism, activated 4,4′-biphenol inhibits topoisomerase II through a protein-interactive mechanism. This was shown by the protein titration assays that, by utilizing increasing amounts of topoisomerase II enzyme in the presence of 100 μM peroxidase-activated 4,4′-biphenol, were able to restore complete enzymatic decatenation activity. In contrast, no recovery was seen with 4,4′-biphenol in the kDNA titration assays. The combination of our DNA and protein titration studies confirm that peroxidase-activated 4,4′-biphenol is capable of inhibiting topoisomerase II via interactions with protein and not through an interaction with DNA. Previous studies have shown that the peroxidase metabolism of 4,4′-biphenol initially results in the almost exclusive formation of diphenoquinone (McGirr et al 1986). Diphenoquinone has previously been shown to bind to protein and not DNA (Subrahmanyam and O'Brien 1985a,b). In order to verify that diphenoquinone was capable of inhibiting human topoisomerase II activity, it was synthesized and tested for inhibitory effects on the enzyme. Inhibition of topoisomerase II by diphenoquinone was seen in vitro at concentrations as low as 10 μM. Our enzyme assay results confirm that diphenoquinone is capable of directly inhibiting human topoisomerase II in vitro and does not require additional metabolic activation.

Interactions between critical proteins and benzene or benzene metabolites have been postulated to be involved in the carcinogenic effects of benzene (Chen and Eastmond 1995b; Creek et al 1997; Irons 1985). This hypothesis is supported by the protein-interactive topoisomerase II inhibitory mechanism of peroxidase-activated 4,4′-biphenol. The topoisomerase II inhibitory effect of 4,4′-biphenol is likely to be attributable to the reactive properties of diphenoquinone, which suggests that the other reactive quinone metabolites of benzene may also be capable of inhibiting topoisomerase II. Support for this mechanism is also found in our isolated enzyme studies, in which a number of peroxidase-activated benzene metabolites were found to be capable of inhibiting human topoisomerase II in vitro. The topoisomerase II enzyme, a target of several quinone chemotherapeutics, is known to contain numerous cysteines (Berger et al 1996; Neder et al 1998; Tsai-Pflugfelder et al 1988). Quinones are also known to have considerable specificity for sulfhydryl groups and bind covalently to cysteine-containing residues in proteins (Hanzlik et al 1994; Monks et al 1992). Binding of quinones to the cysteines

contained within topoisomerase II would be likely to disrupt the function of the enzyme at numerous points in its multistep catalytic cycle (Neder et al 1998).

In addition to showing that benzene and benzene metabolites inhibit the catalytic activity of topoisomerase II in vivo and in vitro, the associated studies in HL-60 cells showed a corresponding decrease in the amount of extractable topoisomerase II in the HL-60 cells exposed to benzene metabolites. The decrease of topoisomerase II activity in vivo and in vitro combined with the associated decrease in topoisomerase II levels seen in benzene metabolite–exposed HL-60 cells is consistent with effects seen with other topoisomerase II inhibitors. The decrease in topoisomerase II protein levels in response to an inhibitor may be explained by a number of possible mechanisms, which include a direct interaction of the benzene metabolites with the topoisomerase II protein or DNA or both, a metabolite-induced block in the cell cycle, or a triggering of a more general apoptotic response in the cells by the metabolites of benzene.

As indicated, a possible mechanism underlying the decrease in topoisomerase II levels induced by the benzene metabolites could be a direct interaction of the bioactive metabolite with either the enzyme or DNA. Topoisomerase II inhibitors such as etoposide and *m*-amsacrine, which form a drug-enzyme-DNA complex, as well as the catalytic inhibitor dexrazoxane (ICRF-187), a bis(dioxopiperazine) compound, have each previously been reported to cause a decrease in the level of extractable topoisomerase II in human cells (Beere et al 1996; Ganapathi et al 1993; Sehested and Jensen 1996; Zwelling et al 1989). The decrease in the functional and extractable topoisomerase II levels has been postulated to be the result of topoisomerase II inhibitors trapping the topoisomerase II on DNA (Ganapathi et al 1993; Sehested and Jensen 1996). These catalytic inhibitors, also referred to as direct inhibitors of topoisomerase II, are thought to lock the protein on the DNA at the postreligation step, whereas topoisomerase II poisons or "cleavable complex"–forming inhibitors stabilize the protein on the DNA after cleavage has occurred and prior to resealing (Sehested and Jensen 1996). Interestingly, intercalating agents, which interfere with topoisomerase II activity by preventing the enzyme from binding DNA, have been shown to actually increase the amount of extractable and functional topoisomerase II in exposed cells (Sehested and Jensen 1996). The topoisomerase II that is trapped on the DNA may also be the target of rapid, protease inhibitor–resistant, proteolytic degradation in human HL-60 cells (Boege et al 1993a,b). As indicated above, the titration and binding studies have shown that topoisomerase II inhibitors formed from bioactivated benzene metabolites work through both direct protein interactions as well as in a DNA-interactive manner. Thus it is possible that the bioactivated benzene metabolites, which act as direct inhibitors of the enzyme and cause a decrease in the extractable topoisomerase II, could act by trapping the enzyme on the DNA and making it a target of proteolytic degradation.

Another possible explanation for the decreased topoisomerase II protein levels induced by the benzene metabolites is a reduction in enzyme levels resulting from a block in the cell cycle. Topoisomerase II inhibitors and benzene have both been shown to block dividing cells at the G_2/M stage (Chen and Beck 1995; Ferguson and Baguley 1994; Irons 1981; Zucker and Elstein 1991). A block in the cell cycle could effectively halt the synthesis of topoisomerase II, ultimately leading to decreased cellular levels. The half-life of topoisomerase II in transformed nonmitotic cells has been reported to be approximately 12 hours, however (Heck et al 1988). Ganapathi and colleagues (1993) reported that 40% of isotopically labeled topoisomerase II was degraded within 4 hours and 80% within 8 hours in HL-60 cells. In synchronized HeLa cells, topoisomerase II protein is present throughout the cell cycle, reaching its lowest levels during G_1 to mid–S phase, then increasing approximately threefold to its highest level in late–S phase until mitosis, after which the cellular levels decrease (Goswami et al 1996). In our studies, the detectable levels of topoisomerase II measured in the metabolite-exposed HL-60 cells decreased to levels that were less than approximately 20% of controls. Thus it appears unlikely that a halt in the cell cycle would account for the decrease seen as a result of the 2-hour cellular exposure to 1,2,4-benzenetriol or hydroquinone. The decrease seen with the 8-hour exposure to 4,4′-biphenol could possibly be explained by a block in the cell cycle. Since the HL-60 cells were not synchronized and given a 30-hour cell cycle, however, a complete block should result in no more than 30% of the cells reaching the G_2/M block during an 8-hour exposure. If we assume a 4-hour to 5-hour life of topoisomerase II and complete degradation of the protein in blocked cells, 8 hours of a cell cycle block should result in approximately a 30% reduction in the total amount of topoisomerase II protein available from all exposed cells (blocked and not blocked) compared with the amount available from the control cells. The topoisomerase II protein levels in the 4,4′-biphenol–exposed cells were decreased by more than 80% compared with levels in control cells. Thus it seems unlikely that a cell cycle block alone would be responsible for the decrease in topoisomerase II protein levels seen in the HL-60 cells. Further investigation would be needed, however, in order to rule out completely the cell cycle

block as a mechanism behind the decrease in the topoisomerase II protein levels.

Another possible explanation for the decrease in topoisomerase II protein levels is the degradation of topoisomerase II via an apoptotic mechanism. Topoisomerase II-α has been shown to be the target of ubiquitin proteolytic degradation during the latent phase of E1A-induced apoptosis (Nakajima 1996; Nakajima et al 1996). Human carcinoma-derived cells with the adenovirus E1A 12S can be induced into apoptosis by treatment with dexamethasone (Nakajima et al 1996). Nakajima and colleagues demonstrated that the topoisomerase II that was immunoprecipitated from the nuclear matrix of these apoptosis-induced cells was polyubiquitinated and degraded more efficiently than that recovered from untreated cells. This degradation was observed prior to the onset of apoptosis and without effects on the topoisomerase II mRNA levels in the exposed cells (Nakajima et al 1996). These findings suggest that a proteolytic-induced decrease in topoisomerase II levels may be an initial event in the apoptotic process. Topoisomerase II inhibitors as well as benzene metabolites have been shown to induce apoptosis in HL-60 cells (Moran et al 1996; Solary et al 1994). Thus it is conceivable that topoisomerase II inhibitors, which are known inducers of an apoptotic response, could also initiate the degradation of topoisomerase II as one of the early events in apoptosis. Although the HL-60 cells exposed to the benzene metabolites were normal in appearance, we can not rule out the possibility that the metabolites of benzene had initiated an apoptotic response in these cells.

The benzene metabolites could also chemically modify topoisomerase II or other proteins that interact with topoisomerase II. Topoisomerase II is thought to interact with more than a dozen proteins that may be required for normal enzyme function (Kroll 1997; Kroll et al 1993). Chemical modification of the topoisomerase II dimer or topoisomerase II–associated proteins could disrupt its ability to interact with other proteins or render the protein nonfunctional and a target for degradation. It is clear that further studies will be required to elucidate the exact mechanism underlying the benzene metabolite–induced decrease in topoisomerase II levels. Regardless of the precise mechanisms behind the benzene and benzene metabolite inhibition of topoisomerase II activity, the significant and reproducible decrease in the topoisomerase II protein levels observed as a result of exposure to benzene metabolites provides a mechanistic explanation for the decreased topoisomerase II activity seen in vitro and in vivo.

Although direct evidence is still lacking, the potential relationship between the inhibition of topoisomerase II by the metabolites of benzene and the development of leukemia is suggested by several lines of evidence. First, exposure to either benzene or chemotherapeutic topoisomerase II inhibitors leads to the development of acute myelogenous leukemia in humans (Aksoy 1988; Anemia 1992; Pedersen-Bjergaard and Rowley 1994). Second, patients who previously received drugs that target topoisomerase II or who were exposed to benzene have developed both myelogenous and monocytic leukemias (Crane et al 1992; Crump 1994; Pedersen-Bjergaard and Rowley 1994; Pui et al 1989). Third, following exposure to benzene, proliferating bone marrow cells exhibit a block at the G_2/M stage in the cell cycle (Irons 1981). A similar effect occurs with topoisomerase II inhibitors, in which cells accumulate at G_2 prior to mitosis (Zucker et al 1991; Zucker and Elstein 1991). Furthermore, both benzene and topoisomerase II inhibitors alter the differentiation profiles of exposed hematopoietic progenitor cells at intermediate stages of differentiation (Kalf and O'Connor 1993).

In addition, certain characteristics of the bone marrow as well as the myelotoxicity exhibited by both benzene and known topoisomerase II inhibitors indicate the plausibility of this mechanism in benzene-induced toxicity. Proliferating cells are, in general, more sensitive to both benzene and topoisomerase II inhibitors than are quiescent cells (Marcus 1987; Sullivan et al 1986, 1987). The high cellular levels of topoisomerase II in proliferating bone marrow cells (Capranico et al 1992), especially in promyelocytic and myelocytic lineages (Kaufmann et al 1991), may explain the high sensitivity seen in these cell lineages following benzene exposure (Kalf and O'Connor 1993; Marcus 1987). Furthermore, the high levels of peroxidase enzymes found in the bone marrow (Kariya et al 1987; Schattenberg et al 1994; Test and Weiss 1986; Twerdok et al 1992; Twerdok and Trush 1988), combined with the observed formation of oxygen radicals (Kolachana et al 1993; Subrahmanyam et al 1991) and the accumulation of phenol in the bone marrow following benzene exposure (Rickert et al 1981), creates conditions that may permit the conversion of phenol and hydroquinone into quinonoid metabolites. The following characteristics (Chen and Eastmond 1995b; Frantz et al 1996) are shared by benzene or its metabolites and chemotherapeutic topoisomerase II inhibitors:

- parent or metabolite has a phenolic or quinonoid structure;
- exhibits increased toxicity to actively dividing cells;
- alters differentiation of immature myeloid cells;
- blocks dividing cells at G_2/M stage;
- yields high frequencies of structural chromosomal alterations; and
- induces acute myelogenous leukemia.

The involvement of topoisomerase II in the formation of chromosomal aberrations was initially proposed by Gaulden (1987). Since that time, topoisomerase II inhibition has been shown to be related to many types of cytological and genetic alterations, including dysfunction in the differentiation of human hemopoietic precursor cells (Francis et al 1994), sister chromatid exchange (Mukherjee et al 1993), chromosomal deletion (Shibuya et al 1994), micronucleus formation (Holmstrom and Winters 1992), polyploidy (Zucker et al 1991; Zucker and Elstein 1991), nonhomologous recombination (Bae et al 1988), and gene amplification (Ikeda 1990). It is interesting to note that most of these genetic alterations have also been reported to be induced by exposure to benzene (Dean 1978, 1985; Snyder and Kalf 1994; Waters et al 1988).

Disrupting topoisomerase II function frequently results in chromosome translocations and deletions, including alterations affecting chromosomes 11q23 and 21q22, which have become hallmarks of treatment-related acute myeloid leukemia induced by topoisomerase inhibitors (Pedersen-Bjergaard and Philip 1991; Pedersen-Bjergaard and Rowley 1994; Smith et al 1994). Translocations have been reported in the blood cells of workers exposed to benzene and in patients who have developed leukemia following exposure to benzene or benzene-containing solvents such as petroleum (Fourth International Workshop on Chromosomes in Leukemia 1984; Li et al 1989; Smith et al 1998; Sole et al 1990; Tasaka et al 1992; Van den Berghe et al 1979). In a number of these cases, the translocations and deletions that have been reported are identical to those that are characteristic of topoisomerase-interactive agents (Table 16). It should be noted, however, that chromosomal alterations similar to those seen following treatment with alkylating chemotherapeutic agents such as loss of all or part of the long arms of chromosomes 5 and 7 have also been reported to occur in benzene-exposed workers and leukemia patients previously exposed to benzene (Pollini and Colombi 1964; Smith et al 1996; Van den Berghe et al 1979; Zhang et al 1998). In addition, trisomy or tetrasomy of a C-group chromosome, occasionally identified as chromosome 8 or 9, has been associated with benzene exposure (Antonucci et al 1989; Eastmond et al 1994; Erdogan and Aksoy 1973; Forni and Moreo 1967; Smith et al 1998; Zhang et al 1996). This suggests that multiple types and mechanisms of genotoxicity may be occurring. Our current working hypothesis is that both the alkylating type of chromosomal alterations (aneuploidy and deletions) as well as the topoisomerase-inhibitor type of alterations (translocations and deletions) are occurring in benzene-exposed individuals. The combination of these types of chromosomal alterations confers an increased risk of leukemia similar to the risk that has been reported for cancer patients who have been treated with both alkylating agents and topoisomerase inhibitors (Smith et al 1994).

Our experimental evidence that demonstrates in vivo and in vitro inhibition of topoisomerase II as a result of benzene or benzene-metabolite exposure, combined with these case reports and the number of shared characteristics between bioactivated benzene and topoisomerase II inhibitors, indicate topoisomerase II could be a critical cellular target of benzene or benzene metabolites. The inhibition of topoisomerase II could be one mechanism involved in benzene-induced toxicity or leukemogenesis. Although we have provided evidence for the role of topoisomerase II inhibition in benzene-induced toxicity, it is also probable that multiple mechanisms are involved in benzene-induced leukemogenesis. Metabolism, transport, damage to other critical cellular targets within hematopoietic cells and stroma, as well as effects on cell proliferation are all likely to play key roles in the leukemic effects of benzene (Smith 1996).

In summary, benzene or its metabolites were shown to inhibit topoisomerase II enzyme activity in an isolated enzyme system, in a human bone marrowderived leukemic cell line, and in an animal model. We have also demonstrated that several known and putative benzene metabolites cause a decrease in the amount of topoisomerase II that can be extracted from exposed human

Table 16. Chromosomal Alterations Characteristic of Topoisomerase II Inhibitors Reported in Leukemia Patients with Previous Exposure to Solvents Containing Benzene

Gender	Age	Chromosomal Alteration	Source of Benzene Exposure	Reference
Male	37	t(8;21)(q22;q22)	Petroleum products	Li et al 1989
Male	36	t(8;21)(q22;q22)	Petroleum products	Li et al 1989
Male	19	Del (11)(q23;q25)	Petroleum products	Li et al 1989
Female	55	t(4;11)(q21;q23)	Solvents	Sole et al 1990
Male	64	t(3;21)(q26.2;q22.1)	Solvents	Tasaka et al 1992

cells compared with controls. These initial results provide valuable support for the hypothesis that inhibition of topoisomerase II contributes to benzene-induced toxicity and leukemogenesis.

PILOT STUDIES IN OCCUPATIONALLY EXPOSED WORKERS*

Due to the long latency period between exposure and the development of cancer as well as other difficulties associated with traditional epidemiologic approaches to identifying human carcinogens, there is an increasing interest in the development of early biological markers of exposure and effect (IARC 1997). Chromosomal alterations have been widely used as an early effect biomarker for the surveillance of human exposure to carcinogenic agents (Carrano and Natarajan 1988; Tucker et al 1997). Increased frequencies of cytogenetic alterations signal that an exposure that is biologically significant and mechanistically related to cancer development has occurred (Sorsa et al 1992). Consistent with this, it has recently been shown that individuals with elevated frequencies of chromosomal aberrations in their peripheral blood lymphocytes are at increased risk for the development of cancer, including leukemia (Bonassi et al 1995; Hagmar et al 1994, 1998).

Previous studies of benzene-exposed workers have frequently shown an association between benzene exposure and elevated frequencies of structural chromosomal aberrations in the peripheral blood lymphocytes of the exposed individuals (Aksoy 1988; Sarto et al 1984). In addition, increased frequencies of numerical aberrations have occasionally been reported to occur in benzene-exposed workers (Aksoy 1988; Eastmond 1993). In contrast to most studies where structural aberrations have been observed in workers with current benzene exposure, however, historically the studies that detected aneuploidy have generally been performed on individuals who had exhibited previous bone marrow toxicity and the studies were initiated some time after exposure had ceased (Ding et al 1983; Forni et al 1971; Liniecki et al 1971; Pollini and Biscaldi 1976; Pollini et al 1969). This suggests that the observed numerical aberrations may be an effect secondary to chronic myelotoxicity or aplastic anemia rather than a direct consequence of benzene exposure.

The detection of structural and numerical aberrations in normal and affected cells has historically been restricted to the manual scoring of metaphase cells. These conventional cytogenetic studies are labor intensive, require highly skilled personnel, and are prone to other technical problems such as chromosomal loss or poor chromosome spreading during metaphase preparation. Due to these limitations, cytogenetic information is generally obtained from only a relatively small number of cells (50 to 100) per individual. In addition, cytogenetic analyses are limited to actively dividing tissues or cells that divide readily in culture. These characteristics make conventional chromosomal analysis difficult to use in routine biomonitoring of occupationally exposed workers and for detecting numerical chromosomal alterations in exposed individuals.

Fluorescence in situ hybridization is a recently developed molecular cytogenetic technique that allows the detection and quantification of both structural and numerical aberrations in metaphase and interphase human cells. Although there are many different applications for FISH in the detection of genetic alterations, the approach that has been most widely applied for human biomonitoring is to use chromosome-specific DNA probes to detect changes in chromosome number in exposed human cells (see Eastmond and Rupa 1995 for a review). This approach is essentially identical to that described in the mouse studies, except that the DNA sequences (probes) commonly used hybridize to centromeric regions of a specific human chromosome rather than to the subcentromeric regions targeted by the mouse probes. The number of copies of that chromosome in the interphase cell is determined by simply counting the number of hybridization regions in the nucleus. This approach has been successfully applied to identify basal and elevated levels of numerical chromosome alterations in various cell types of human populations (Martin et al 1997; Ramirez et al 1997; Robbins et al 1995, 1997; Surralles et al 1997a; Zhang et al 1996).

We have recently developed a new multicolor FISH strategy to identify hyperdiploidy more accurately in interphase cells and to distinguish these cells from nuclei containing breaks affecting the labeled regions (Eastmond and Rupa 1995; Eastmond et al 1994, 1995; Rupa et al 1995). This approach uses a classical-satellite probe that hybridizes to the large pericentric heterochromatin region of chromosome 1 combined with a second α-satellite probe, which is labeled with a different fluorochrome that hybridizes to an adjacent centromeric region. By evaluating the number and location of the colored hybridization regions in the interphase nucleus, hyperdiploidy for chromosome 1 can be distinguished from breakage within the heterochromatic region or between the two labeled

* The publication of the Estonian population study includes F Marcon, A Zijno, R Crebelli, A Carere, T Veidebaum, and K Peltonen as coauthors. When published, the Chinese worker studies will include N Rothman, RB Hayes, M Dosemeci, L Zhang, MT Smith, W Bechtold, S Yin, and G Li as coauthors.

regions. A diagram of this tandem labeling approach is shown in Figure 21. Additional tandem probe combinations have been developed to allow alterations affecting human chromosomes 9 (9cen-9q12) and 16 (16cen-16q11.1) also to be detected (Hasegawa et al 1995; Schuler et al 1998). Subsequent studies using cells treated with chemicals or radiation in vitro, as well as cells obtained from chemically exposed humans, have shown that this technique can be effectively used to detect hyperdiploidy and breakage affecting these heterochromatic regions in the treated cells or exposed individuals (Conforti-Froes et al 1997; Rupa and Eastmond 1997; Rupa et al 1995, 1997; Schuler et al 1998; Surralles et al 1997b). Although the term *breakage* is used throughout this section, a sizable portion (~10%–40%) of these alterations represent translocations, inversions, and other types of potentially stable chromosome exchanges (Rupa et al 1995).

The objective of this portion of the project was to apply this new tandem FISH approach to determine whether it could be effectively used to monitor structural and numerical alterations in benzene-exposed workers. Samples were obtained from two groups of investigators studying cytogenetic alterations in benzene-exposed groups. Blood smears and 48-hour cultured lymphocytes collected from workers at a shale oil petrochemical plant in Kohtla-Järve, Estonia, were obtained from Drs Angelo Carere and Riccardo Crebelli of the Italian Institute of Health in Rome, Italy. These samples were collected as part of a larger collaborative research effort to develop biomarkers of occupational and environmental exposure. Preparations of 72-hour cultured lymphocytes were obtained from a study of benzene-exposed workers in China conducted by Drs Martyn Smith and Nathaniel Rothman as part of a joint study between the US National Cancer Institute and the Chinese Academy of Preventive Medicine. For the Estonian samples, analysis using the tandem labeled probes for chromosomes 1 or 9 was conducted on the blood smear G_0 lymphocytes and polymorphonuclear cells (granulocytes) and on the 48-hour cultured lymphocytes. For the Chinese samples, the tandem probes for chromosome 1 were applied to the 72-hour lymphocyte slides.

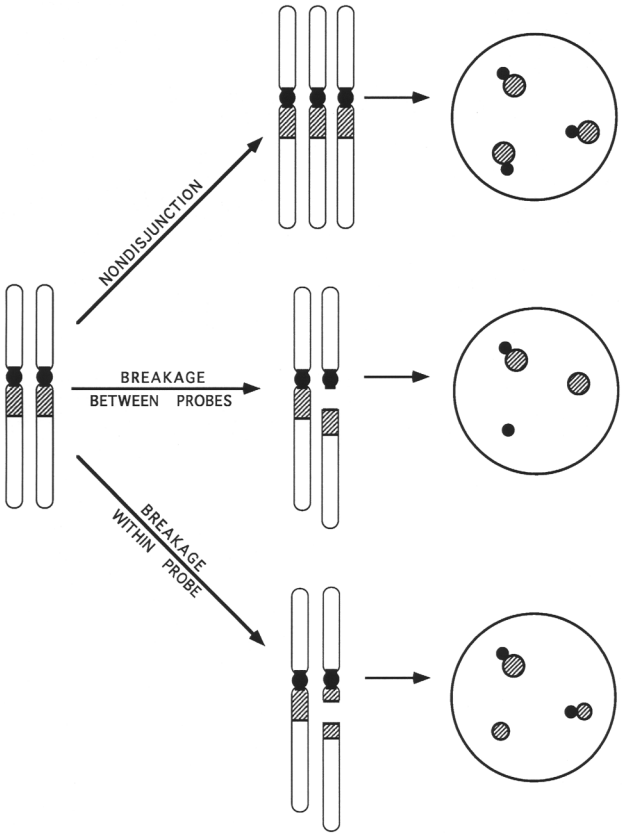

Figure 21. A schematic illustration of the hybridization strategy using two adjacent DNA probes to detect chromosomal breakage and hyperdiploidy in metaphase and interphase cells. For better visualization, hybridization areas have been enlarged and do not reflect the actual size of the DNA probes. (Adapted from Rupa et al 1995; reprinted with permission from the American Association for Cancer Research.)

METHODS

Study Population: Estonian Group

Seventeen workers from a shale oil plant in Kohtla-Järve (Estonia), together with eight unexposed donors from Iisaku rural village (Estonia), were enrolled in the study in the late summer of 1994. Shale oil workers were engaged in benzene production (12 individuals) and in coke oven operations (5 individuals). All subjects completed a questionnaire about personal information including smoking and drinking habits, health status, and age.

Blood samples were obtained by venipuncture during autumn of 1994. From each sample, several blood smears were prepared as follows: 10 μL of whole blood was placed onto a glass slide, smeared, air-dried, and fixed with cold methanol for 20 min. After air drying, slides were stored at −20°C under a nitrogen atmosphere.

Lymphocyte cultures were established by adding 0.5 mL heparinized whole blood to 4.5 mL RPMI 1640 medium with 25 mM Hepes buffer and L-glutamine (Gibco, Scotland), supplemented with 20% heat-inactivated fetal calf serum (Hyclone) and antibiotics (Flow), 2% phytohemagglutinin (PHA) (HA-15; Murex, Italy) and incubated for 48 hours at 37°C. Afterward, cells were treated with a 0.075 M KCl hypotonic solution for 10 min at 37°C, fixed three times in methanol:acetic acid (3:1), and stored at −20°C until slides were prepared and used for in situ hybridization (up to 1 year).

Study Population: Chinese Groups

Slides containing Carnoy-fixed 72-hour cultured lymphocytes collected as part of a joint study between the US National Cancer Institute and the Chinese Academy of Preventive Medicine were obtained from Drs Martyn Smith and Nathaniel Rothman. The study population, sampling procedures, and cell-culturing methods for these samples have been previously described (Rothman et al 1996a, 1997; Zhang et al 1996).

Probes, Probe Generation, and Labeling Conditions

Detailed protocols of probes, labeling, and hybridization conditions for the tandem labeling procedure for chromosomes 1 and 9 as well as the principle underlying this technique are described in detail elsewhere (Hasegawa et al 1995; Rupa et al 1995). A digoxigenin-labeled α-satellite probe for chromosome 1 (D1Z5; Oncor, Gaithersburg MD) to label the centromeric region and a Cy3-labeled classical-satellite probe (pUC 1.77; Cooke and Hindley 1979; Tagarro et al 1994) to target the adjacent pericentric heterochromatin region were used for all hybridization procedures. The labeled probe for the classical-satellite region of chromosome 1 was prepared using the nick-translation protocol provided with the DNA polymerase/DNAse 1 enzyme mixture (GIBCO-BRL) and Cy3-dUTP (Amersham Life Science, Arlington Heights IL) as label.

The α-satellite probe for chromosome 9 was generated by polymerase chain reaction using genomic DNA and oligonucleotide primers. A single 24mer primer, AL9-3, 5′-CCT GAA AGC GCT TAA AAC GTC GTC CGC-3′ (OPERON Technologies, Alameda CA), for the α-satellite region of chromosome 9 was chosen from the published sequence of that region (Rocchi et al 1991). The template used was DNA-isolated by standard methods (Davis et al 1986) from the human/rodent somatic cell hybrid GM10611, a hamster cell line containing human chromosome 9 (NIGMS Human Genetic Mutant Cell Repository: Coriell Institute for Medical Research, Camden NJ). As template for the chromosome 9–specific classical-satellite probe, we used plasmid DNA isolated from the pHuR98 clone containing a classical-satellite III sequence of human chromosome 9 inserted into the pBR322 vector at the Pst I site (Moyzis et al 1987; ATCC, Rockville MD). The primers used for PCR of the plasmid DNA were sequences of pBR322 flanking the Pst I site. The primers used were HuR98-2 (5′-GGA ACC GGA GTC GAA TGA AGC CAT-3′) and HuR98-3 (5′-AGT AAG TAG TTC GCC AGT TAA TAG-3′), which were synthesized by OPERON Technologies (Alameda CA). PCR conditions are described in detail in Hasegawa and colleagues (1995). Briefly, after hot-starting the reaction by denaturing the DNA at 94°C for 8 min and then adding 5 U of *Tfl* polymerase (Epicenter Technologies, Madison WI) under the oil layer, amplification and labeling was performed for 25 cycles of 30 seconds at 94°C, 1 min at 55°C (Csat3 annealing temperature was 37°C), and 2 min at 72°C by PCR. A final extension step at 72°C was carried out for 15 min. PCR amplification products were then labeled by nick-translation as described above using Cy3-dUTP as label for the classical-satellite probe and digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis IN) or FluoroGreen (Amersham, Arlington Heights IL).

Fluorescence in Situ Hybridization

For all in situ hybridizations, standard conditions without further pretreatment of slides were tested initially (Rupa et al 1995). Using this method, hybridization quality sufficient to allow accurate scoring could not be obtained for either of the two studies. After testing different pretreatment conditions using combinations of proteases and detergents, the following standard pretreatment procedures were used for the different cell types. In some cases, particularly for the Chinese slides, this involved rehybridizing the previously hybridized slides. For the blood smears from the Estonian study, the red blood cells were removed using three treatments for 5 min each of Carnoy fixative (methanol:acetic acid, 3:1), the cells dehydrated in 70%, 85%, and 100% ethanol for 2 min each at room temperature, and hybridized using the standard conditions. The 48-hour cultured lymphocytes from the same study and the 72-hour cultured lymphocytes from the Chinese study were incubated in 0.1% (w/v) saponine (Sigma) for 30 min at room temperature and briefly rinsed with phosphate-buffered saline (PBS). The slides were then treated with pepsin (1 μg/mL in 0.01 N HCl) for 15 min at room temperature. After rinsing the slides briefly with PBS, slides were further treated with proteinase K (1 μg/mL in 10 mM Tris [pH 7.5], 10 mM EDTA, and 150 mM NaCl) for 10 min at 37°C. Finally, slides were dehydrated in 70%, 85%, and 100% ethanol for 2 min each at room temperature.

All hybridization procedures were then performed using previously described procedures (Trask and Pinkel 1990). Briefly, slides were immersed in a 70% formamide-2× SSC solution for 5 min at 70°C, dehydrated in an ethanol series as described earlier, and placed on a slide warmer at 37°C. Multicolor FISH with α-satellite probes and classical-satellite probes for chromosome 1 (or 9) were performed in 55% formamide, 10% dextran sulfate, 1× SSC using 1 μg sheared herring sperm DNA, 1 μL of digoxigenin-labeled α-satellite probe for chromosome 1 (or 10 to 50 μg each of FluoroGreen- and digoxigenin-labeled α-satellite probe for chromosome 9), 20 to 100 ng digoxigenin-labeled

classical-satellite probe for chromosome 1 (or 9), and ddH_2O as required, all in a volume of 10 μL. Posthybridization washes were performed in 2× SSC for 5 min at room temperature, three times for 4 min each in 0.1× SSC at 65°C, and one time in PX buffer (0.1 M phosphate buffer, pH 8.0; 0.5% [w/v] Triton-X-100) for 5 min at room temperature. The digoxigenin-labeled α-satellite probes for chromosomes 1 and 9 were detected using a mouse anti–digoxigenin immunoglobulin G (IgG) (Boehringer Mannheim; 3.2 μg/mL in PX buffer with 5% nonfat dry milk [PXM buffer]), followed by an amplification round with digoxigenin-conjugated sheep anti–mouse antibody (Boehringer Mannheim; 20 μg/mL in PXM) and a third layer consisting of FITC-conjugated sheep anti–digoxigenin IgG (Boehringer Mannheim; 20 μg/mL in PXM). To counterstain the DNA, 4′,6-diamidino-2-phenylindole (0.1 μg/mL in phenylenediamine antifade) was used.

All slides were scored using a Nikon fluorescence microscope at ×1,250 magnification. The frequencies of alterations were determined by scoring 1,000 cells per individual from coded slides for the cultured lymphocyte samples and 500 cells per cell type for the blood smear mononuclear and polymorphonuclear cells. Only slides with acceptable hybridization quality were scored. Fluorescence filters and scoring criteria for the tandem labeling technique were described earlier (Eastmond et al 1994). A triple-band-pass filter (Chroma Technology, Brattleboro VT, #P/N 61002) was used to visualize the yellow-green (FITC; α-satellite), red (Cy3; classical-satellite), and blue (DAPI; DNA counterstain) simultaneously. In the case of a cell with more than two red signals or a cell with a wide separation between the yellow-green α-satellite signals and the red classical-satellite region, the signals were verified by changing to a filter optimal for the individual fluorochrome: a blue filter (Nikon B-2A; excitation at 450 to 490 nm, emission at 520 nm) for the FITC signals and a green filter (Chroma Technology, Brattleboro VT, #31004; excitation at 540 to 580 nm, emission at 600 to 660 nm) for the Cy3 signals. Hybridization regions comprised of a Cy3-labeled hybridization region (classical-satellite probe) adjacent to a somewhat smaller yellow spot (α-satellite probe) were scored as indicating the presence of an intact chromosome 1 or 9. However, a nucleus containing three hybridization regions in which two were comprised of adjacent red and yellow fluorochromes and a third region containing only a Cy3-labeled region was scored as containing two copies of that chromosome with a breakage event having occurred within the chromosomal region targeted by the Cy3-labeled classical-satellite probe. In addition, a wide separation between the regions labeled by the α- and classical-satellite probes were scored as breakage between the hybridization regions targeted by the DNA probes. Finally, hybridization regions appearing as doublets or diffused signals were scored as a single hybridization region.

Statistical Analyses

The breakage and hyperdiploidy results from the Estonian workers were analyzed using two basic approaches. The breakage data, with a square-root transformation applied to improve normality, was generally analyzed using a variety of parametric approaches including simple and multiple regression, ANOVA, the PFLSD test, and a Student *t* test. Due to a large number of 0 values in the Estonian data, the nonparametric Kruskall-Wallis ANOVA, Spearman rank correlation, and Mann-Whitney *U* test were used to compare the hyperdiploidy results.

For the Chinese study groups, following square-root transformation, analyses were performed using simple and multiple regression, ANOVA, the PFLSD test, and a *t* test. The urinary metabolites were transformed using the natural logarithm +1 transformation prior to their use for regression analysis (Zhang et al 1996). All tests were performed using the StatView SE+Graphics statistical software (Abacus Concepts, Berkeley CA).

RESULTS

Benzene-Exposed Estonian Workers

A detailed assessment of benzene exposure in the Kohtla-Järve shale oil workers, showing significantly higher levels of all exposure markers in exposed workers compared with control individuals, has been published elsewhere (Kivisto et al 1997). Data on the subset of workers enrolled in this study are summarized in Table 17. The highest individual values of exposure markers were consistently detected among benzene factory workers. Because of the high variability and the small size of the study groups, however, only the difference among blood benzene values in exposed and control subjects attained statistical significance ($P \leq 0.05$; Kruskall-Wallis ANOVA).

The tandem labeling FISH analysis was performed on both granulocytes and unstimulated lymphocytes in the blood smears and on the 48-hour cultured lymphocytes. The results of tandem labeling analysis of chromosome 1 in blood smears are summarized in Figures 22 and 23. Breakages in the labeled region of chromosome 1 were generally higher in granulocytes than in unstimulated lymphocytes ($P \leq 0.05$; paired *t* test). In comparing the benzene factory workers, the coke oven workers, and control group, an analysis of breakage frequencies using ANOVA

Table 17. Main Characteristics of the Exposed and Control Individuals Enrolled in the Cytogenetic Survey at the Shale Oil Petrochemical Plant in Kohtla-Järve, Estonia[a]

	Control Subjects (n = 8)	Benzene Factory Workers (n = 12)	Coke Oven Operation Workers (n = 5)
Age (years)	40.6 ± 11.6	34.4 ± 11.8	38.0 ± 14.6
Employment length (months)	—	79.1 ± 69.8	136.7 ± 163.4
Smoking (pack years)	7.9 ± 8.4 (n = 5)	10.7 ± 10.4 (n = 9)	4.8 ± 8.7 (n = 2)
Air benzene (mg/m^3)	—	4.1 ± 8.0	1.1 ± 0.5
Blood benzene (nmol/L)	22.4 ± 10.0	85.9 ± 115.3	53.6 ± 18.2
Urinary *t,t*-muconic acid (μmol/L)	1.2 ± 1.9	21.7 ± 44.8	5.1 ± 3.6
Urinary *S*-phenylmercapturic acid (μg/g creatinine)	2.6 ± 3.1	39.7 ± 64.9	12.9 ± 11.0

[a] Data from Kivisto et al (1997); presented as means ± SD.

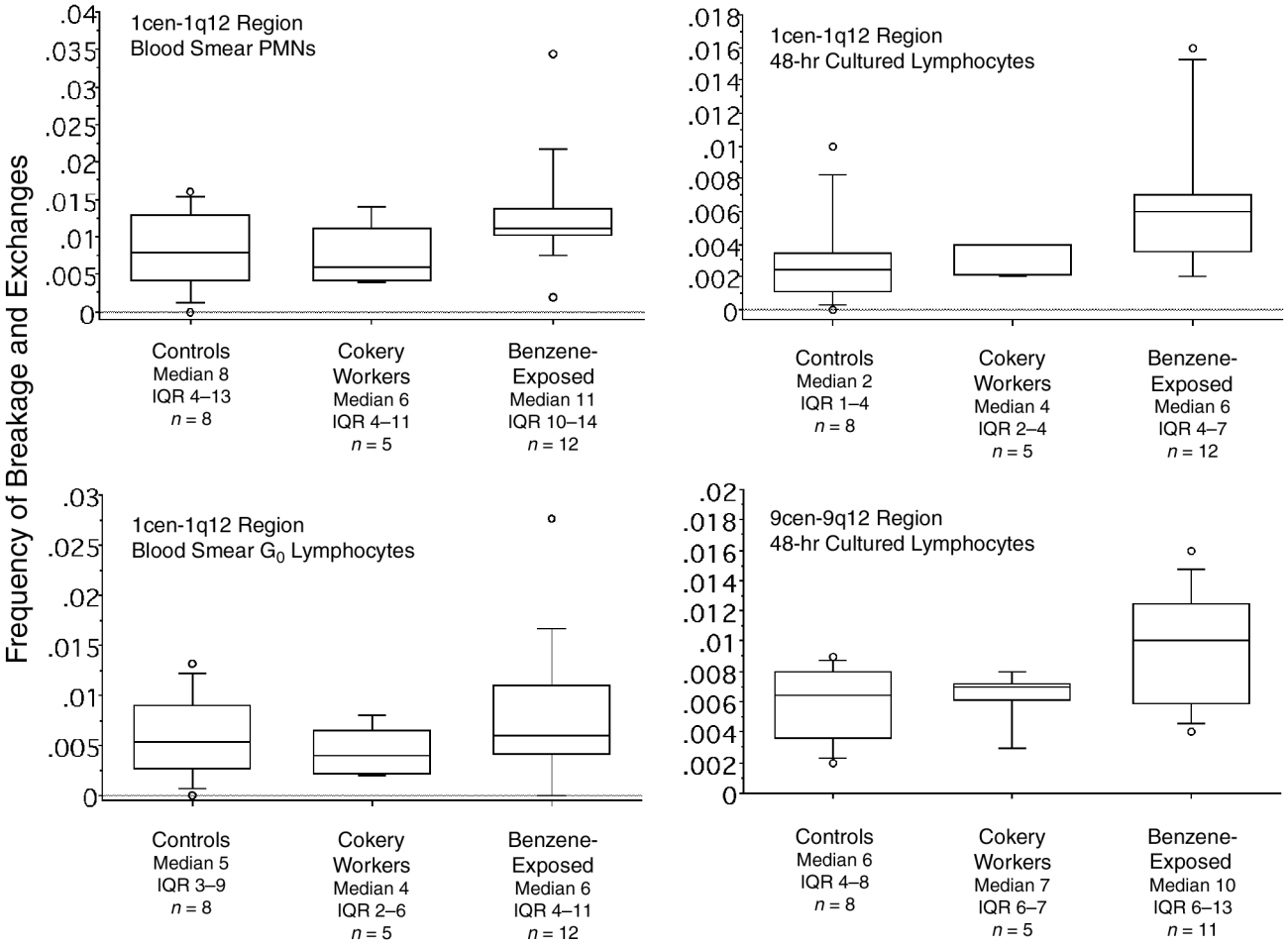

Figure 22. Frequency of breakage and exchanges affecting either the 1cen-1q12 region or the 9cen-9q12 region in cells from the Estonian study groups. Values for medians and IQRs are × 10^{-3}.

and the Kruskall-Wallis test indicated that the differences among the three groups were not statistically significant (Figure 22). Similarly, no significant association was observed between (a) the incidence of breakage in either the lymphocytes or granulocytes and (b) age, smoking habits, benzene-exposure markers, or length of employment; the only exception was a significant association observed between breakage in the 1q12 region and smoking ($P = 0.027$; t test). It is doubtful, however, whether this was biologically significant given the number of comparisons and the observation that the frequency in the nonsmokers was higher than that in the smokers. For the blood smears, significant differences in chromosome 1 hyperploidy (Figure 23) were not seen between the two cell types or among the study groups. The frequencies of 1q12 breakage in the granulocytes were significantly correlated with those detected in the G_0 lymphocytes ($P \leq 0.05$; regression). The association, however, was largely due to a single benzene-exposed individual who exhibited high breakage frequencies in both granulocytes and G_0 lymphocytes.

The tandem labeling analysis of chromosomes 1 and 9 in cultured lymphocytes, harvested 48 hours after stimulation, showed a modest increase of breakage in the cells from benzene production workers compared with both nonexposed control subjects and coke oven workers exposed to lower benzene levels (Figure 22). The median frequencies of breaks in the 1cen-1q12 region were 2‰, 4‰, and 6‰ in control subjects, coke oven workers, and benzene workers, respectively. A significant difference in the frequencies was seen using either ANOVA or the Kruskall-Wallis test and was due to a significant difference between the benzene-exposed and the control group ($P \leq 0.05$; PFLSD). The median incidences of breaks affecting the 9cen-9q12 region were 6‰, 7‰, and 10‰ in the control

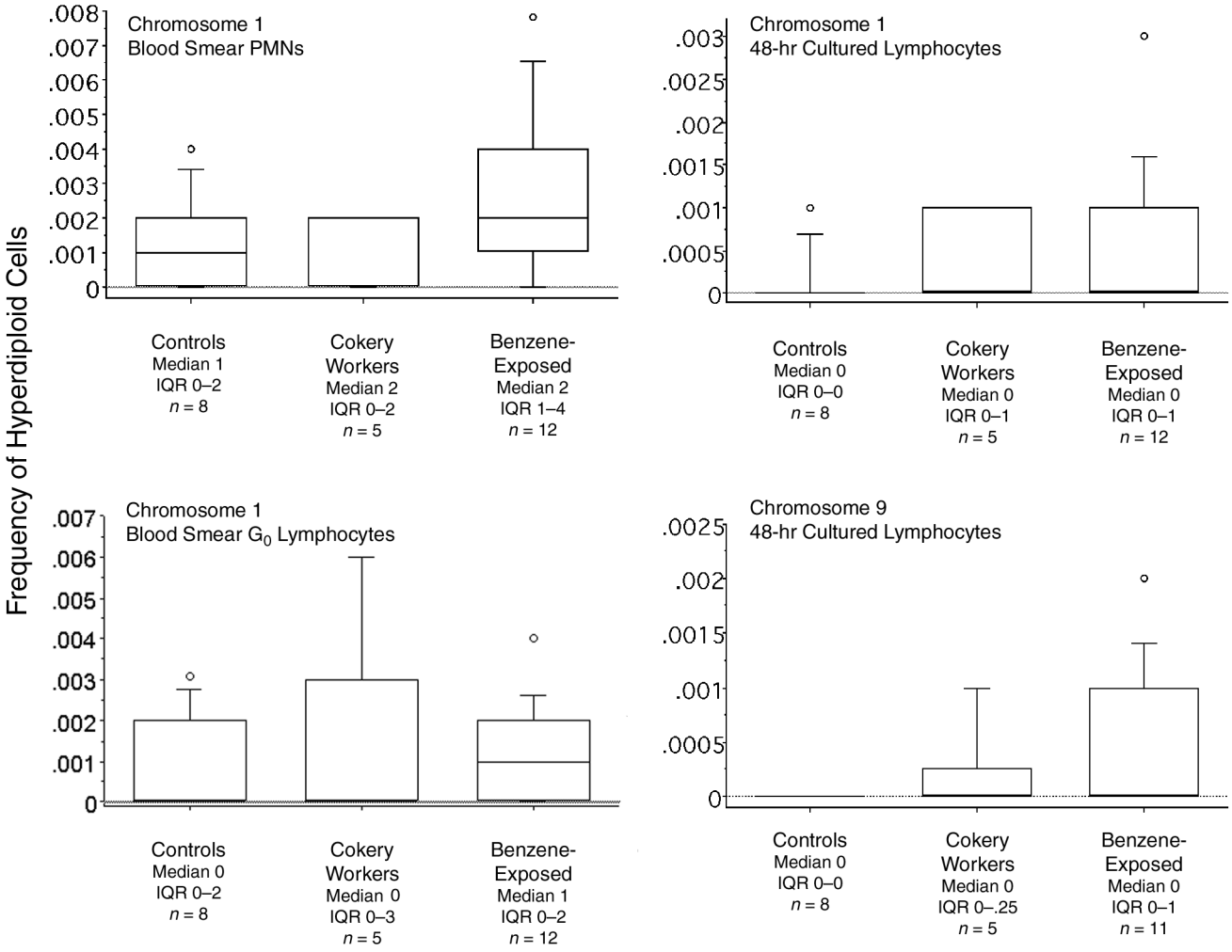

Figure 23. Frequency of cells hyperdiploid for chromosome 1 or 9 from the Estonian study groups. Values for medians and IQRs are $\times 10^{-3}$.

subjects, coke oven workers, and benzene workers, respectively. Although the difference between the chromosome 9 breakage for three groups did not quite attain statistical significance ($P = 0.053$; Kruskall-Wallis test), an excess of breaks was observed in comparing the benzene-exposed workers with the control group using the Mann-Whitney U test ($P \leq 0.05$). No correlation was seen between (a) breakage in either 1q12 or 9q12 regions and (b) the exposure biomarkers, age, or smoking status, however. The frequency of breakage observed in the 9cen-9q12 region was significantly higher than the frequency of breaks in the 1cen-1q12 region when compared across all groups ($P < 0.001$), possibly reflecting a difference in susceptibility of the two targeted regions to breakage (Brogger 1977; Meyne et al 1979).

In spite of this difference, an analysis of individual results revealed a strong linear correlation between the results obtained with the two chromosomes ($P < 0.001$; Figure 24), adding confidence to the overall reliability of results and the reproducibility of the method. This was encouraging considering that the hybridizations were performed for different chromosomes, using different probes, and scored independently in two laboratories. (The chromosome 1 analyses for the 48-hour cultured lymphocytes were performed in Rome, Italy, whereas the chromosome 9 analyses for these cells was carried out in Riverside CA.) In the stimulated lymphocytes, the incidence of hyperploidy for both chromosomes was slightly higher in the benzene-exposed workers compared with the other groups (Figure 23). The differences did not attain statistical significance

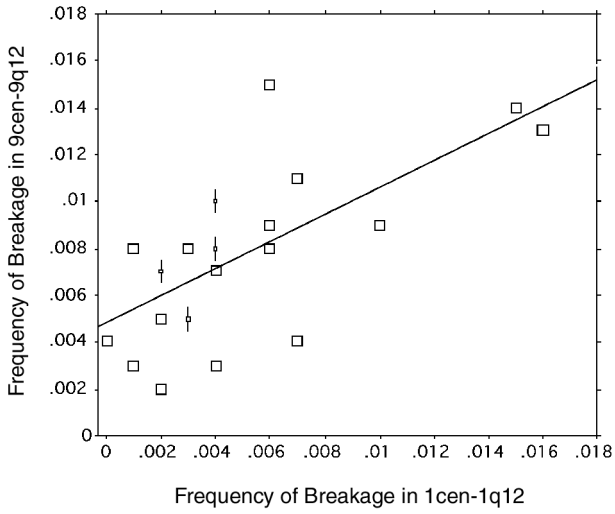

Figure 24. Correlation between breakage affecting the heterochromatin of chromosomes 1 and 9 in 48-hour cultured lymphocytes from the Estonian study groups. $Y = 0.0048 + 0.5758X$; $r^2 = 0.445$. Individual data points are shown as open squares; a small box with two lines indicates two points at the same position.

for either chromosome, again possibly reflecting the small sample sizes and the overall low frequency of hyperploidy of chromosomes 1 and 9 observed (0.64‰—that is, 16 out of 25,000 scored cells). Again, no association was seen between hyperdiploidy and the exposure biomarkers. It should be noted that only 1 cell hyperdiploid for chromosome 1 (out of 10 total observed), and 0 cells hyperdiploid for chromosome 9 (out of 6 total observed), were detected in the 8,000 cells of control subjects. This suggests a possible effect of occupational exposure. The frequencies among the exposed are within the hyperdiploidy range seen in our previous studies, however (Eastmond et al 1995).

Chinese Workers with Current Benzene Exposure

Details on subject enrollment and population characteristics as well as various biological effects observed in the Chinese worker study have been previously published (Rothman et al 1996a,b, 1997; Zhang et al 1996). In brief, 44 healthy control subjects and 44 benzene-exposed workers matched by age, sex, and from the same area of Shanghai, China, were enrolled in the study. Key characteristics of the current exposure study groups are shown in Table 18. As described by Rothman and associates (1996a), the benzene exposures were quite high: The median exposure concentration in the exposed group was 31 ppm (8-hour time-weighted average [TWA]) with a measured range from 1.6 to 328.5 ppm. The median benzene exposure in the control subjects was 0.02 ppm, with a range from 0.01 to 0.1 ppm. Clear and significant differences in benzene exposure variables were seen between the two groups (Table 18).

Fluorescence in situ hybridization with the tandem labeled probes for chromosome 1 was performed on the 72-hour cultured interphase lymphocytes from the workers and control subjects. Significant differences were not seen for either endpoint ($P > 0.05$). The median frequency of breakage affecting the 1cen-q12 region in the control subjects was 2‰ with an interquartile range (IQR) of 1‰ to 3‰. An almost identical frequency was seen in the exposed workers with a median of 2‰ (IQR 1‰–4‰). Similar results were seen for hyperdiploidy. The median frequency of hyperdiploidy in the cells from the benzene-exposed workers and the control subjects was 2 with an IQR of 1‰ to 4‰. When the exposed group was divided into two exposure categories, one group with exposures below the median of 31 ppm and a second group with exposures above 31 ppm, no difference from the control subjects was seen with either hyperdiploidy or breakage in workers from either of the exposure categories (Figure 25). The median (and IQR) frequencies of breakage were 2‰

Table 18. Key Characteristics of the Control and Currently Benzene-Exposed Chinese Workers from Shanghai, China[a]

	Controls (n = 44)	Benzene-Exposed (n = 44)
Gender		
Male	23	23
Female	21	21
Age (years)	34.8 (32.4–39.3)	34.8 (30.0–39.6)
Smoking status		
Nonsmoker	23	23
Smoker	21	21
Cigarettes/day	10 (10–16.3)	10 (5.8–20)
Pack years	8.5 (3.0–13.3)	6.5 (3.1–11.6)
Urinary phenol (μg/g creatinine)	17.3 (8.4–27.3)	91.5 (36.8–343)
Urinary *t,t*-muconic acid (μg/g creatinine)	0.18 (0.16–0.23)	26.1 (7.9–50.1)

[a] Data are presented as medians (IQR).

(1‰–3‰), 2‰ (1‰–4‰), 2‰ (0‰–4‰) in the control, low-exposure, and high-exposure groups, respectively. The median (and IQR) frequencies for hyperdiploidy among these same groups were 2‰ (1‰–4‰), 2‰ (0‰–3‰), and 3‰ (2‰–5‰), suggesting that a slight increase in hyperdiploid cells might be occurring in the workers with the highest exposure.

If real, this pattern would be similar to that reported by Zhang and coworkers in which a significant increase in hyperdiploid cells was seen in the highly exposed group when analyzed using FISH with a single chromosome 9 probe (Zhang et al 1996). In this earlier study, the authors reported that a significant correlation between urinary phenol and hyperdiploidy was seen among the exposed workers. Based on this result, a similar comparison was performed for the chromosome 1 tandem label data. A significant correlation between urinary phenol and hyperdiploidy for chromosome 1 was seen for the exposed workers (Figure 26: $P = 0.0075$; $r = 0.412$) as well as the entire study population ($P = 0.0146$; $r = 0.322$). A similar association between the frequency of cells hyperdiploid for chromosome 1 and urinary *t,t*-muconic acid concentration for both the exposed group (Figure 26; $P = 0.0046$; $r = 0.434$) and the entire study group ($P = 0.0415$; $r = 0.271$). No association between urinary phenol or *t,t*-muconic acid was seen for breakage whether the anal-

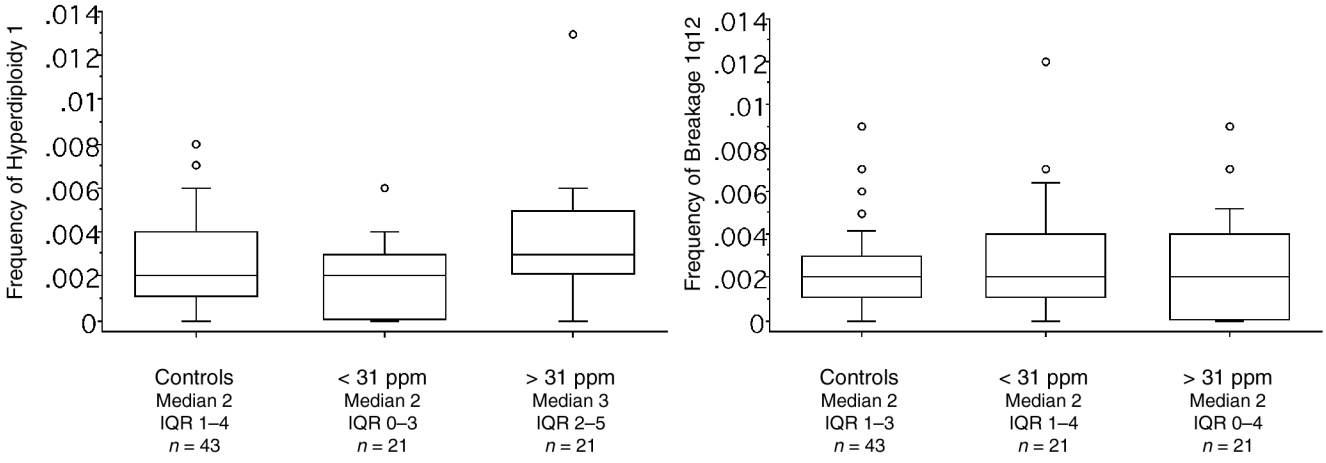

Figure 25. Frequency of (top) hyperdiploidy for chromosome 1 and (bottom) breakage affecting the 1cen-1q12 region in 72-hour cultured lymphocytes from the currently exposed Chinese workers. Values for medians and IQRs are $\times 10^{-3}$.

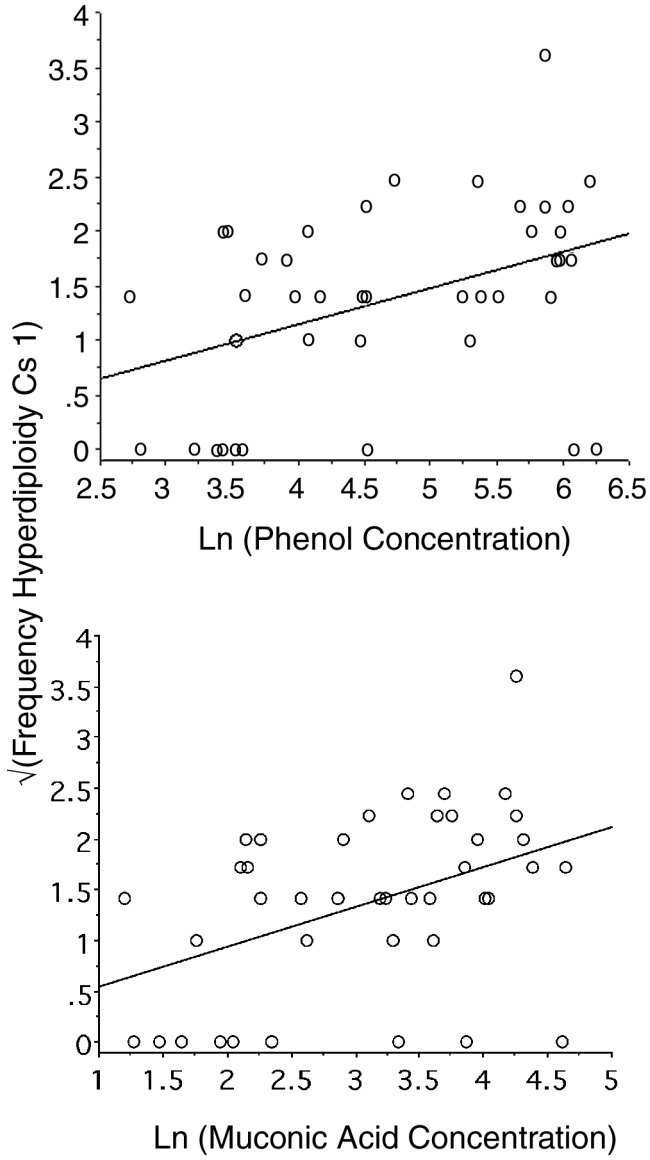

Figure 26. Linear regression between the square root–transformed frequencies of chromosome 1 hyperdiploidy and the ln + 1–transformed (A) urinary phenol concentration (Y = 0.181 + 0.334X; r^2 = 0.17) **and (B) urinary *t,t*-muconic acid concentration** (Y = 0.161 + 0.393X; r^2 = 0.19) **among the benzene-exposed Chinese workers.**

ysis was based upon the entire study group or only the exposed group. Significant associations also were not observed between (a) the frequency of breakage or hyperdiploidy and (b) age, smoking status, or cigarettes per day. A weak but statistically significant difference in the breakage frequency was observed between the males and females, however (P = 0.038; t test). The mean (± SD) frequency of breakage in the male cells (1.485 ± 0.821) was higher than that seen in cells from the females (1.116 ± 0.789).

Chinese Workers Previously Poisoned by Benzene

As part of the joint study between the US National Cancer Institute and the Chinese Academy of Medicine, 50 workers with evidence of previous benzene poisoning were identified and agreed to participate in a series of follow-up studies on cancer risk, genetic susceptibility, and persistent genetic damage. Previous reports have described the results of these studies on cancer risk and genetic susceptibility (Hayes et al 1997; Rothman et al 1997). It should be noted that, for the most part, the previously poisoned workers had not been exposed to benzene for several years prior to sample collection. Fifty individuals matched for age and sex who worked in a sewing machine manufacturing facility or an administrative facility in Shanghai were selected as control subjects. Key characteristics of the study groups are presented in Table 19.

As previously, FISH with the tandem probes for chromosome 1 was used to identify the frequency of breakage and exchanges affecting the 1cen-1q12 region and hyperdiploidy for chromosome 1 in the previously poisoned workers compared with the control subjects. No significant increase in hyperdiploidy or breakage was seen in the cultured lymphocytes of the poisoned workers compared with control subjects (Figure 27). The median frequency of breakage in the 1cen-q12 region in control subjects was 2‰ with an IQR of 1‰ to 3.5‰. An almost identical frequency was seen in the poisoned workers with a median of 2‰ (IQR 1‰–3‰). Similar results were seen for hyperdiploidy. The median frequency of hyperdiploidy for chromosome 1 in the cells of the poisoned workers was 3‰ (IQR 1‰–4‰), whereas it was 2‰ (IQR 1‰–3.5‰) in the control subjects, a difference that was not significant. Significant associations were not observed between the frequency of

Table 19. Key Characteristics of Control and Previously Benzene-Poisoned Chinese Workers from Shanghai, China[a]

	Controls (n = 50)	Benzene-Poisoned (n = 50)
Gender		
Male	23	23
Female	27	27
Age (years)	41.0 (37.6–54)	44.0 (38.2–58.8)
Smoking status		
Nonsmoker	30	32
Smoker	18	17
Cigarettes/day	13 (10–20)	6 (2–12)
Pack years	12.9 (7.3–22)	7 (2.6–11.1)

[a] Data are presented as medians (IQR).

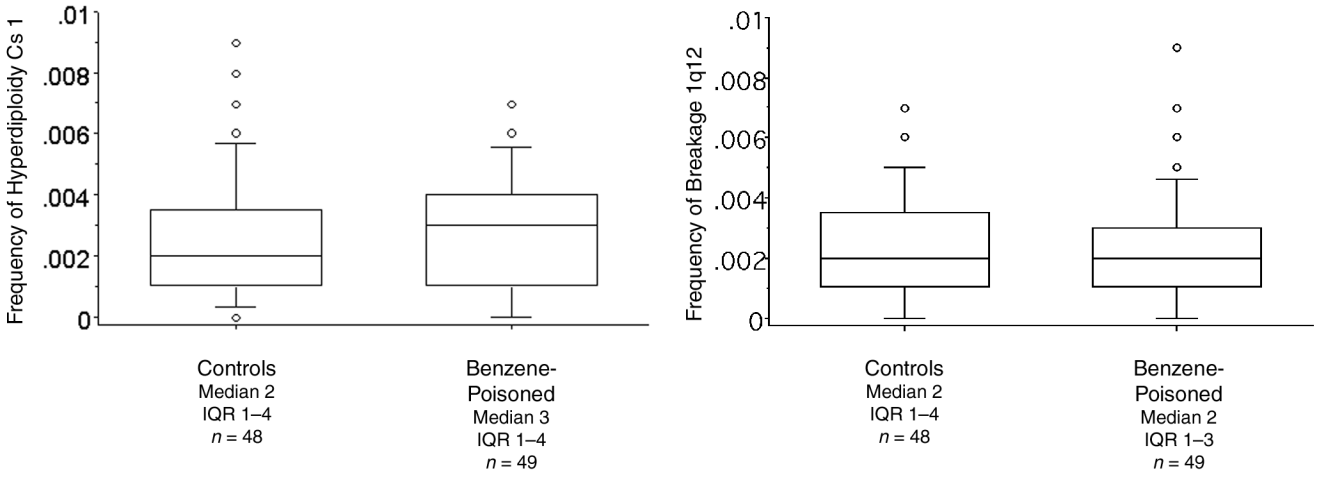

Figure 27. Frequency of (left) hyperdiploidy for chromosome 1 and (right) breakage affecting the 1cen-1q12 region of 72-hour cultured lymphocytes from the previously poisoned Chinese workers. Values for medians and IQRs are $\times 10^{-3}$.

breakage or hyperdiploidy for cigarettes per day, pack years, sex, current benzene exposure, alcoholic drinks per week, or historical benzene-exposure levels. A significant association between breakage frequency and age was observed ($P = 0.015$; regression), with breakage and exchanges increasing with age.

DISCUSSION

The objective of these studies was to determine whether this new tandem FISH approach could be used effectively to monitor structural and numerical alterations in benzene-exposed workers. Our results clearly show it is feasible to apply FISH to studies of benzene-exposed workers and provide some indication of the value of this technique for human biomonitoring. The modest nature of the effects seen and the inconsistency between the study groups highlight some of the problems that can be encountered in applying FISH or another relatively new biomarker to a human population.

In the Estonian workers, a significant increase in breakage within the chromosome 1 heterochromatin and a marginally significant increase within the 9 heterochromatin was seen in the cultured lymphocytes of the exposed workers compared with those of the control subjects. A weak tendency toward increased hyperdiploidy was also seen in these workers. The exposure concentrations of these individuals were relatively low, averaging between 1 and 2 ppm. In contrast, no increase in breakage was detected among either the currently exposed Chinese workers or those who had previously experienced a benzene poisoning episode. This was somewhat surprising in that the exposures among the Chinese workers were much higher—20-fold or more—than those of the Estonian workers. This suggests that the breakage seen in the Estonian study was either induced by another agent in the workplace (such as polynuclear aromatic hydrocarbons), difference in lifestyle factors (such as diet or medications), or possibly that there is an unusual relation between exposure and response at this endpoint. The strong correlation between the chromosome 1 and 9 breakage data obtained by the two laboratories in the Estonian study provides considerable support that the increase in aberrations was real and not simply a result of random fluctuations.

The difference in breakage between the Estonian and Chinese groups may also be due in part to the differences in harvest time. The Estonian lymphocytes were harvested at 48 hours, a time routinely used and optimized for detecting structural alterations, whereas the Chinese lymphocytes were harvested at 72 hours, when most of the cells should be in their second metaphase. The latter time point is preferable for detecting aneuploidy, particularly that occurring in vitro, but is less efficient for detecting breaks because chromosome fragments can be lost as the cell cycles from the first to the second mitosis (Carrano and Natarajan 1988).

In the study of the currently exposed Chinese workers, no significant association was seen between hyperdiploidy and benzene exposure when using current measurements of benzene in the air. When an individual's personal exposure and ability to metabolize benzene was considered, however, significant correlations between (a) urinary phenol and *t*,*t*-muconic acid and (b) hyperdiploidy were seen. These results are consistent with those previously seen for this population using a chromosome 9 probe (Zhang et al 1996). In the previous study, a significant association between the benzene-exposed workers and control subjects was not detected until the workers were classified by exposure category. As seen with our results,

the correlation between hyperdiploidy and exposure was substantially improved by the use of urinary metabolites to classify individual exposures. These results are also consistent with our mouse studies, in which only a modest increase in hyperdiploidy was detected in the treated mice. These results highlight the importance of personal metabolism and exposure measurements in human biomonitoring studies, particularly when studying relatively weak effects.

One of the challenges in interpreting the hyperdiploidy and breakage results of the benzene-exposed workers is the somewhat variable nature of the frequencies of hyperdiploidy and breakage that can be observed, particularly in the control cells. For example, the median frequency of hyperdiploidy observed in the Chinese worker control subjects was quite consistent: 2‰ (IQR 1‰–4‰) for the currently exposed group and 2‰ (IQR 1‰–3.5‰) for the control subjects in the benzene-poisoned study. In contrast, the chromosome 1 hyperdiploidy frequencies among the control subjects in the Estonian study was 0‰ (IQR 0‰–0‰) in the 48-hour cultured lymphocytes, 0‰ (IQR 0‰–2‰) among the G_0 lymphocytes, and 1‰ (IQR 0‰–2‰) in the granulocytes. Similarly, the frequency of chromosome 9 hyperdiploidy was 0‰ (IQR 0‰–0‰) in the 48-hour cultured lymphocytes. Although similar, the Estonian values are somewhat lower than those seen for the Chinese workers and are lower than those seen previously in other studies from our laboratory (Rupa et al 1995) as well as those expected from the literature (Eastmond et al 1995). The control frequencies reported by Zhang and coworkers, however, are considerably higher, with mean hyperdiploid frequencies of 7‰ in control subjects (Zhang et al 1996). It is not certain if these differences reflect differences in the involvement of specific chromosomes; differences in scoring criteria, cell culture, hybridization efficiency; or other technical considerations.

One important observation from these studies is the requirement for high-quality slides for the tandem FISH. The slides that we obtained for each of the human studies, although collected and stored for FISH analysis, posed significant challenges to obtain hybridizations of sufficient quality for tandem FISH. From our experience, the purity of the methanol and acetic acid used to fix the cells is critical to the success of the hybridizations. In addition, the humidity when preparing the slides and the time in storage seem to influence hybridization efficiency and quality. This is an important consideration for human biomonitoring in developing nations, because reagent quality at harvest can significantly influence hybridizations performed later. For each of the groups of slides, modifications of the standard hybridization procedures, including the use of detergents, proteases, and rehybridization of slides, had to be developed to obtain successful hybridizations. As part of the Chinese study, we attempted to use tandem labeling on blood smears from the study groups. Unfortunately, these had not been stored for FISH analysis and our efforts to obtain scorable hybridizations were unsuccessful. As a result of the required modifications in methods, the differences seen from cell type to cell type or study to study may be influenced by technical differences as well as true biological effects. The comparisons between control and exposed subjects in an experiment, however, should be valid since the slides were processed together and scored in a blinded fashion.

In summary, these studies demonstrated the feasibility of using FISH with tandem DNA probes to detect chromosome alterations in interphase granulocytes, G_0 lymphocytes, and cultured lymphocytes obtained from benzene-exposed workers. Although we were able to use FISH on samples from both the Estonian and the Chinese workers, the results of the two studies were somewhat inconsistent. In the Estonian workers with lower exposures and smaller sample size, the benzene-exposed workers exhibited higher frequencies of breakage than control subjects. A trend toward increased hyperdiploidy was also seen, although the frequencies in the exposed workers were low and within the range of our historical control frequencies. In the more highly exposed and larger Chinese worker study, no increase in breakage affecting the 1q12 region was seen among the exposed workers. A trend toward increased hyperdiploidy of chromosome 1 was seen in the exposed workers when the concentration of urinary benzene metabolites was used in conjunction with the frequency of hyperdiploidy in the individual workers. The weak increase in hyperdiploidy detected in the exposed workers is consistent with the mouse data in the section describing our second specific aim and indicates that benzene is a weak inducer of aneuploidy in vivo.

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APPENDIX A. Viability of Human HL-60 Cells Exposed to Phenolic Metabolites of Benzene With and Without Hydrogen Peroxide for Different Time Periods

Table A.1. Percent Viability of Human HL-60 Cells Exposed to Phenol Without and With Hydrogen Peroxide[a]

Phenol (μM) or Control Treatment	Duration of Treatment (hours)				
	2	4	8	24	48
Phenol Without H_2O_2					
No treatment	97 ± 2	96 ± 2	98 ± 1	99 ± 1	98 ± 1
0.1% DMSO	98 ± 2	98 ± 1	97 ± 2	99 ± 1	96 ± 2
10	97 ± 1	97 ± 1	98 ± 1	98 ± 1	99 ± 1
50	98 ± 1	96 ± 2	97 ± 2	98 ± 1	96 ± 2
100	97 ± 3	99 ± 1	98 ± 2	97 ± 0	96 ± 3
500	99 ± 1	98 ± 1	97 ± 2	96 ± 3	98 ± 1
1,000	96 ± 2	97 ± 1	98 ± 2	97 ± 3	95 ± 6
Phenol With 1 μM H_2O_2					
0.1% DMSO	99 ± 2	99 ± 1	99 ± 0	98 ± 0	96 ± 3
10	98 ± 0	99 ± 2	98 ± 1	96 ± 2	98 ± 1
50	98 ± 1	98 ± 2	98 ± 3	96 ± 4	98 ± 0
100	95 ± 1	99 ± 1	96 ± 3	99 ± 1	97 ± 1
500	98 ± 1	97 ± 2	96 ± 0	97 ± 1	96 ± 2
1,000	97 ± 1	98 ± 1	98 ± 1	98 ± 1	96 ± 1
Phenol With 10 μM H_2O_2					
0.1% DMSO	98 ± 1	99 ± 1	97 ± 1	98 ± 1	98 ± 1
10	98 ± 1	97 ± 3	97 ± 2	96 ± 3	96 ± 2
50	97 ± 3	96 ± 2	99 ± 2	99 ± 1	96 ± 4
100	97 ± 2	98 ± 2	98 ± 1	96 ± 2	96 ± 2
500	98 ± 1	98 ± 2	99 ± 1	97 ± 1	98 ± 2
1,000	97 ± 1	98 ± 1	99 ± 0	97 ± 0	97 ± 1
Phenol With 20 μM H_2O_2					
0.1% DMSO	97 ± 2	97 ± 2	98 ± 1	95 ± 3	94 ± 2
10	98 ± 2	96 ± 3	95 ± 2	94 ± 2	94 ± 1
50	98 ± 2	99 ± 1	96 ± 2	94 ± 3	96 ± 3
100	96 ± 4	98 ± 2	96 ± 3	93 ± 4	94 ± 2
500	97 ± 1	95 ± 2	97 ± 1	93 ± 2	97 ± 2
1,000	97 ± 2	96 ± 3	97 ± 1	97 ± 1	94 ± 3

[a] Data are the mean percent viablilty of three experiments ± SD.

Table A.2. Percent Viability of Human HL-60 Cells Exposed to 2,2′-Biphenol Without and With Hydrogen Peroxide[a]

2,2′-Biphenol (μM) or Control Treatment	Duration of Exposure (hours)				
	2	4	8	24	48
2,2′-Biphenol Without H_2O_2					
No treatment	98 ± 2	98 ± 1	98 ± 3	98 ± 1	97 ± 2
0.1% DMSO	100 ± 1	99 ± 1	99 ± 0	96 ± 1	98 ± 1
10	98 ± 2	99 ± 1	98 ± 3	98 ± 2	98 ± 1
50	97 ± 1	94 ± 6	98 ± 1	98 ± 0	97 ± 1
100	97 ± 1	97 ± 1	97 ± 2	98 ± 1	96 ± 1
500	92 ± 4	87 ± 11	87 ± 3	85 ± 5	55 ± 9
1,000	91 ± 6	85 ± 8	82 ± 11	32 ± 15	6 ± 8
2,2′-Biphenol With 1 μM H_2O_2					
0.1% DMSO	97 ± 1	99 ± 1	98 ± 1	99 ± 1	98 ± 2
10	97 ± 2	98 ± 1	98 ± 2	97 ± 1	97 ± 1
50	96 ± 3	98 ± 2	98 ± 1	98 ± 1	97 ± 1
100	96 ± 3	97 ± 2	95 ± 1	96 ± 3	96 ± 1
500	85 ± 3	90 ± 3	87 ± 4	76 ± 10	63 ± 9
1,000	89 ± 8	87 ± 15	88 ± 8	20 ± 3	1 ± 9
2,2′-Biphenol With 10 μM H_2O_2					
0.1% DMSO	98 ± 1	99 ± 1	97 ± 3	97 ± 1	98 ± 1
10	98 ± 2	98 ± 1	97 ± 2	97 ± 3	98 ± 2
50	98 ± 1	98 ± 2	99 ± 1	97 ± 2	97 ± 2
100	97 ± 3	98 ± 1	98 ± 1	98 ± 2	95 ± 2
500	93 ± 3	91 ± 2	88 ± 6	83 ± 12	65 ± 15
1,000	85 ± 6	89 ± 1	89 ± 6	12 ± 6	2 ± 2
2,2′-Biphenol With 20 μM H_2O_2					
0.1% DMSO	96 ± 0	98 ± 1	98 ± 1	96 ± 3	97 ± 1
10	97 ± 1	98 ± 2	98 ± 3	97 ± 1	95 ± 3
50	98 ± 1	97 ± 1	95 ± 2	97 ± 2	97 ± 2
100	95 ± 2	97 ± 2	97 ± 1	96 ± 2	93 ± 5
500	90 ± 5	91 ± 2	90 ± 2	68 ± 17	62 ± 10
1,000	81 ± 10	77 ± 15	69 ± 15	6 ± 6	4 ± 6

[a] Data are the mean percent viability of three experiments ± SD.

Table A.3. Percent Viability of Human HL-60 Cells Exposed to 4,4′-Biphenol Without and With Hydrogen Peroxide[a]

4,4′-Biphenol (μM) or Control Treatment	Duration of Exposure (hours)				
	2	4	8	24	48
4,4′-Biphenol Without H_2O_2					
No treatment	95 ± 5	97 ± 2	94 ± 1	97 ± 2	97 ± 2
0.1% DMSO	98 ± 1	98 ± 2	99 ± 1	96 ± 1	95 ± 1
10	98 ± 2	98 ± 1	96 ± 3	95 ± 4	98 ± 1
50	93 ± 10	98 ± 1	97 ± 1	97 ± 2	94 ± 3
100	97 ± 2	98 ± 1	96 ± 4	95 ± 2	64 ± 7
500	98 ± 2	97 ± 2	92 ± 7	32 ± 24	1 ± 1
1,000	98 ± 1	97 ± 2	87 ± 12	22 ± 26	0 ± 0
4,4′-Biphenol With 1 μM H_2O_2					
0.1% DMSO	97 ± 3	96 ± 3	96 ± 0	97 ± 2	96 ± 2
10	98 ± 1	98 ± 1	97 ± 1	96 ± 2	97 ± 3
50	96 ± 4	98 ± 3	95 ± 4	93 ± 6	86 ± 14
100	92 ± 8	98 ± 2	97 ± 1	83 ± 4	61 ± 23
500	90 ± 12	97 ± 1	89 ± 5	28 ± 22	0 ± 0
1,000	88 ± 15	95 ± 3	86 ± 14	10 ± 5	0 ± 0
4,4′-Biphenol With 10 μM H_2O_2					
0.1% DMSO	97 ± 1	96 ± 4	97 ± 2	97 ± 1	97 ± 4
10	97 ± 4	97 ± 1	97 ± 2	96 ± 2	95 ± 2
50	98 ± 1	98 ± 3	97 ± 3	96 ± 2	95 ± 1
100	99 ± 1	95 ± 3	94 ± 3	90 ± 4	53 ± 27
500	96 ± 1	96 ± 6	87 ± 9	14 ± 5	0 ± 0
1,000	96 ± 1	98 ± 2	85 ± 14	7 ± 7	0 ± 0
4,4′-Biphenol With 20 μM H_2O_2					
0.1% DMSO	97 ± 1	96 ± 4	96 ± 3	94 ± 5	97 ± 2
10	97 ± 3	97 ± 2	94 ± 3	94 ± 2	93 ± 3
50	97 ± 2	99 ± 2	95 ± 1	95 ± 3	95 ± 1
100	95 ± 8	93 ± 10	94 ± 4	85 ± 2	57 ± 20
500	89 ± 8	89 ± 6	76 ± 19	12 ± 4	14 ± 25
1,000	96 ± 1	85 ± 6	64 ± 30	2 ± 3	0 ± 0

[a] Data are the mean percent viability of three experiments ± SD.

Table A.4. Percent Viability of Human HL-60 Cells Exposed to 1,2,4-Benzenetriol Without and With Hydrogen Peroxide[a]

1,2,4-Benzenetriol (μM) or Control Treatment	Duration of Exposure (hours)				
	2	4	8	24	48
1,2,4-Benzenetriol Without H_2O_2					
No treatment	99 ± 1	98 ± 1	99 ± 0	99 ± 2	98 ± 2
0.1% DMSO	99 ± 2	98 ± 1	97 ± 2	97 ± 3	97 ± 1
10	97 ± 2	97 ± 2	99 ± 1	98 ± 2	97 ± 1
50	96 ± 5	92 ± 1	79 ± 10	63 ± 13	53 ± 14
100	90 ± 1	59 ± 13	51 ± 24	32 ± 15	4 ± 2
500	83 ± 8	68 ± 3	17 ± 8	1 ± 2	0 ± 0
1,000	88 ± 2	65 ± 24	47 ± 7	3 ± 3	0 ± 0
1,2,4-Benzenetriol With 1 μM H_2O_2					
0.1% DMSO	99 ± 1	99 ± 1	99 ± 2	96 ± 1	98 ± 1
10	99 ± 1	98 ± 2	97 ± 3	97 ± 2	99 ± 2
50	97 ± 3	88 ± 7	78 ± 5	59 ± 8	56 ± 27
100	83 ± 4	77 ± 13	43 ± 24	19 ± 11	3 ± 4
500	70 ± 19	67 ± 4	38 ± 16	3 ± 3	1 ± 1
1,000	79 ± 1	64 ± 32	29 ± 3	3 ± 2	0 ± 0
1,2,4-Benzenetriol With 10 μM H_2O_2					
0.1% DMSO	98 ± 2	98 ± 1	98 ± 1	98 ± 3	99 ± 1
10	98 ± 1	98 ± 1	96 ± 1	93 ± 5	98 ± 1
50	94 ± 3	84 ± 4	67 ± 10	53 ± 18	40 ± 22
100	83 ± 13	69 ± 17	54 ± 7	29 ± 10	7 ± 5
500	75 ± 17	53 ± 35	28 ± 16	2 ± 2	0 ± 0
1,000	74 ± 20	65 ± 15	19 ± 12	1 ± 2	0 ± 0
1,2,4-Benzenetriol With 20 μM H_2O_2					
0.1% DMSO	98 ± 1	99 ± 1	95 ± 6	94 ± 3	97 ± 3
10	100 ± 1	96 ± 2	96 ± 2	92 ± 6	93 ± 7
50	87 ± 12	87 ± 11	64 ± 23	50 ± 24	28 ± 9
100	82 ± 19	57 ± 18	34 ± 11	10 ± 16	2 ± 2
500	83 ± 9	63 ± 10	28 ± 11	2 ± 3	0 ± 0
1,000	74 ± 15	55 ± 19	20 ± 8	1 ± 1	0 ± 0

[a] Data are the mean percent viability of three experiments ± SD.

Table A.5. Percent Viability of Human HL-60 Cells Exposed to Catechol Without and With Hydrogen Peroxide[a]

Catechol (μM) or Control Treatment	Duration of Exposure (hours)				
	2	4	8	24	48
Catechol Without H_2O_2					
No treatment	97 ± 1	96 ± 2	96 ± 2	95 ± 4	96 ± 2
0.1% DMSO	98 ± 2	98 ± 2	97 ± 2	95 ± 4	96 ± 3
10	98 ± 1	95 ± 6	93 ± 6	98 ± 2	96 ± 4
50	97 ± 1	98 ± 1	94 ± 2	93 ± 5	94 ± 2
100	98 ± 2	98 ± 2	91 ± 7	70 ± 21	48 ± 4
500	96 ± 1	96 ± 2	62 ± 4	4 ± 5	0 ± 0
1,000	96 ± 1	91 ± 8	32 ± 28	0 ± 1	0 ± 0
Catechol With 1 μM H_2O_2					
0.1% DMSO	98 ± 2	98 ± 2	98 ± 1	97 ± 1	96 ± 3
10	97 ± 2	99 ± 2	95 ± 4	96 ± 4	95 ± 1
50	95 ± 5	97 ± 3	96 ± 1	91 ± 5	91 ± 2
100	98 ± 2	97 ± 0	91 ± 4	68 ± 24	50 ± 16
500	96 ± 2	94 ± 8	56 ± 35	2 ± 3	0 ± 0
1,000	94 ± 2	82 ± 22	28 ± 27	1 ± 2	0 ± 0
Catechol With 10 μM H_2O_2					
0.1% DMSO	98 ± 2	97 ± 2	93 ± 1	97 ± 2	96 ± 1
10	97 ± 2	96 ± 2	94 ± 5	97 ± 2	96 ± 4
50	97 ± 2	97 ± 1	94 ± 1	93 ± 4	88 ± 13
100	97 ± 3	98 ± 1	92 ± 3	61 ± 25	38 ± 36
500	97 ± 2	96 ± 2	65 ± 23	6 ± 8	0 ± 0
1,000	95 ± 5	86 ± 14	25 ± 33	0 ± 0	0 ± 0
Catechol With 20 μM H_2O_2					
0.1% DMSO	97 ± 1	97 ± 1	93 ± 1	91 ± 5	96 ± 3
10	96 ± 2	97 ± 1	93 ± 2	93 ± 5	95 ± 3
50	97 ± 1	98 ± 1	92 ± 5	91 ± 2	89 ± 3
100	94 ± 6	95 ± 2	88 ± 5	51 ± 8	28 ± 25
500	97 ± 2	90 ± 3	39 ± 26	0 ± 0	0 ± 0
1,000	95 ± 4	82 ± 20	26 ± 16	1 ± 2	0 ± 0

[a] Data are the mean percent viability of three experiments ± SD.

Table A.6. Percent Viability of Human HL-60 Cells Exposed to Hydroquinone Without and With Hydrogen Peroxide[a]

Hydroquinone (μM) or Control Treatment	Duration of Exposure (hours)				
	2	4	8	24	48
Hydroquinone Without H_2O_2					
No treatment	94 ± 4	96 ± 4	99 ± 2	95 ± 2	99 ± 2
0.1% DMSO	96 ± 2	94 ± 4	97 ± 2	97 ± 1	98 ± 1
10	98 ± 2	98 ± 2	94 ± 6	98 ± 1	98 ± 1
50	97 ± 2	91 ± 14	9 ± 8	0 ± 1	0 ± 0
100	96 ± 2	69 ± 42	15 ± 25	0 ± 0	0 ± 0
500	71 ± 11	17 ± 4	3 ± 3	0 ± 1	0 ± 0
1,000	58 ± 20	18 ± 6	6 ± 4	0 ± 1	0 ± 0
Hydroquinone With 1 μM H_2O_2					
0.1% DMSO	98 ± 2	98 ± 1	95 ± 5	98 ± 1	97 ± 1
10	97 ± 3	96 ± 3	96 ± 0	96 ± 1	95 ± 3
50	98 ± 2	72 ± 37	19 ± 28	1 ± 2	0 ± 0
100	96 ± 3	57 ± 46	7 ± 9	0 ± 0	0 ± 0
500	63 ± 22	24 ± 21	3 ± 2	1 ± 2	0 ± 0
1,000	27 ± 25	11 ± 8	5 ± 3	1 ± 2	0 ± 0
Hydroquinone With 10 μM H_2O_2					
0.1% DMSO	97 ± 0	98 ± 1	99 ± 1	97 ± 1	98 ± 1
10	96 ± 1	96 ± 2	94 ± 6	96 ± 2	96 ± 1
50	98 ± 2	44 ± 47	6 ± 9	0 ± 1	0 ± 0
100	88 ± 9	29 ± 35	3 ± 4	1 ± 1	0 ± 0
500	50 ± 31	7 ± 4	3 ± 3	3 ± 3	0 ± 1
1,000	21 ± 14	7 ± 6	5 ± 2	5 ± 4	1 ± 1
Hydroquinone With 20 μM H_2O_2					
0.1% DMSO	99 ± 1	98 ± 2	98 ± 1	95 ± 3	98 ± 1
10	96 ± 3	81 ± 27	76 ± 38	75 ± 37	73 ± 36
50	86 ± 12	14 ± 20	1 ± 1	1 ± 1	0 ± 0
100	75 ± 21	18 ± 20	0 ± 1	0 ± 1	0 ± 0
500	29 ± 17	9 ± 10	3 ± 3	6 ± 7	0 ± 1
1,000	14 ± 7	15 ± 20	3 ± 1	3 ± 5	0 ± 0

[a] Data are the mean percent viability of three experiments ± SD.

ABOUT THE AUTHORS

David A Eastmond is currently chair of the environmental toxicology graduate program at the University of California, Riverside. He received his PhD from the University of California, Berkeley, and his MS and BS degrees from Brigham Young University in Provo UT. In 1987, he was selected as an Alexander Hollaender Distinguished Postdoctoral Fellow and, for the following two years, conducted postdoctoral research at Lawrence Livermore National Laboratory. Shortly thereafter, Dr Eastmond joined the faculty at University of California, Riverside, where he is actively involved in teaching and research. Dr Eastmond's laboratory focuses on the mechanisms involved in the toxicity and carcinogenesis of environmental chemicals. His research has centered on the metabolism and chromosome-damaging effects of benzene, a widely used industrial chemical and environmental pollutant, and *ortho*-phenylphenol, a commonly used fungicide and disinfectant. Dr Eastmond has worked on developing and applying new molecular techniques such as fluorescent in situ hybridization to rapidly assess chromosomal damage caused by environmental and occupational chemicals in human populations.

Maik J Schuler received his PhD in food chemistry and environmental toxicology from the University of Kaiserslautern, Germany, in 1994, and his diploma in biology from the same university. He is currently a senior research scientist in the genetic toxicology group within the drug safety evaluation department at Pfizer. His research interests are in the mechanisms underlying the formation of numerical and structural chromosomal aberrations in cells.

Christopher Frantz received a PhD in environmental toxicology from the University of California, Riverside in 1998, an MA in biological sciences with specialization in toxicology from San Jose State University, and a BS in animal science from the University of California, Davis. He is currently a toxicologist in the biopharmaceutical development division at Stanford Research International in Menlo Park CA, with a primary focus on preclinical drug development. His research interests include molecular mechanisms of toxicity and carcinogenicity.

Hongwei Chen received his PhD in environmental toxicology from the University of California, Riverside in 1994, his MS in insect toxicology from the Shanghai Institute of Entomology, Chinese Academy of Science, and a BS from East China Normal University. He is currently a senior research scientist in the department of toxicology at Bristol-Myers Squibb. His research interests include molecular mechanisms of chemical mutagenicity and carcinogenicity and risk assessment based on the mechanisms of toxicity.

Robert Parks received his BS in biology from University of California, Riverside, and was subsequently certified as a clinical lab specialist in cytogenetics (CLSpCG). He is currently a clinical laboratory cytogenetic technologist at the University of California, Davis, Medical Center.

Ling Wang received her MS degree in cell biology from the Institute of Cell Biology at Xiamen University in People's Republic of China in 1993, and a BS in microbiology from the same university. She is currently a PhD candidate in environmental toxicology at the University of California, Riverside. Her research focuses on cellular and molecular mechanisms of genotoxicity induced by environmental chemicals.

Leslie Hasegawa received her MS degree in plant sciences in 1978 and a BSc in biochemistry in 1975, both from the University of California, Riverside. She is currently a staff research associate in Dr Eastmond's laboratory.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Marcon F, Zijno A, Crebelli R, Carere A, Veidebaum T, Peltonen K, Parks R, Schuler M, Eastmond D. 1999. Chromosome damage and aneuploidy detected by interphase multicolour FISH in benzene-exposed shale oil workers. Mutat Res 445:155–166.

Frantz CE, Chen H, Eastmond DA. 1996. Inhibition of human topoisomerase II in vitro by bioactive benzene metabolites. Environ Health Perspect 104(Suppl 6):1319–1323.

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ABBREVIATIONS AND OTHER TERMS

‰	per thousand (cells)
AMS	accelerator mass spectrometry
ANOVA	analysis of variance
ANU sucrose	sucrose prepared by the Australian National University
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
AUC	area under the curve

BSA	bovine serum albumin
Cyp2e1	rodent cytochrome P450 2E1 gene
DAPI	4′,6-diamidino-2-phenylindole
ddH_2O	double deionized H_2O
DMSO	dimethyl sulfoxide
ECL-plus	immuno-enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
GPA	glycophorin A
GSH	glutathione
H_2O_2	hydrogen peroxide
HBSS	Hanks balanced salt solution
Hprt	rodent hypoxanthine-guanine phosphoribosyl transferase gene
HRP	horseradish peroxidase
IARC	International Agency for Research on Cancer
IgG	immunoglobulin G
IPCS	International Programme on Chemical Safety
IQR	interquartile range
KCl	potassium chloride
kDa	kilodaltons
kDNA	kinetoplast DNA
M+m+	micronuclei containing both major (M) and minor (m) satellites
M+m−	micronuclei containing only the major satellite
M−m+	micronuclei containing only the minor satellite
M−m−	micronuclei failing to hybridize either major or minor satellite
MeOH	methanol
NaCl	sodium chloride
NCE	normochromatic erythrocyte
NQO1	NAD(P)H:quinone oxidoreductase 1 gene
NTP	National Toxicology Program
PBS	phosphate-buffered saline
PCE	polychromatic erythrocyte
PCR	polymerase chain reaction
PFLSD	protected Fisher least significant difference
PHA	phytohemagglutinin
PMN	polymorphonuclear cells
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidenedifluoride
PXM	PX buffer with nonfat dry milk
SCE	sister chromatid exchange
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	standard saline citrate
Tfl	*Thermus flavus*
TWA	time-weighted average

INTRODUCTION

The development of simple, sensitive, and specific analytic assays is critical for assessing the risk of low-level exposure to benzene in humans. HEI funded research by Dr David Eastmond and colleagues to develop two connected approaches to address this issue. The first approach involved detecting chromosomal alterations (see the sidebar, which explains many of the cytogenetic and molecular biological terms used in the Commentary and Investigators' Report) in cells from benzene-exposed mice and from humans occupationally exposed to benzene. Eastmond proposed to use a modification of a molecular cytogenetic technique known as fluorescence in situ hybridization (FISH)*, which is described in detail below. This approach, if successful, may be better than other cytogenetic methods for estimating benzene's effects because it (1) has the potential to be sensitive, (2) allows information to be obtained from many cell types, and (3) may be useful in large population studies. It could also provide information about how different chromosomal alterations arise. Furthermore, HEI's Research Committee thought that Eastmond's proposal to measure chromosomal alterations in both mice and occupationally exposed humans would provide useful data to compare benzene's effects in different species.

Dr Eastmond's second approach was to investigate whether the effects of benzene or its metabolites on DNA were indirect, acting through the inhibition of the enzyme topoisomerase II, which plays a key role in maintaining chromosomal structure. This part of the study was expected to provide novel information about mechanisms relevant to the carcinogenic effects of benzene, which are not well understood.

Eastmond's study was funded under RFA 93-1, "Novel Approaches to Extrapolation of Health Effects for Mobile-Source Toxic Air Pollutants."† His draft Investigators' Report underwent external peer review; the HEI Health Review Committee discussed the report and the reviewers' critiques, and prepared this Commentary. The Commentary is intended to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations, and placing the report in scientific perspective.

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

†Dr Eastmond's 3-year study, *Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans,* began in November 1994. Total expenditures were $500,800. The draft Investigators' Report from Eastmond and colleagues was received for review in June 1998. A revised report, received in June 1999, was accepted for publication in August 1999. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Commentary.

SCIENTIFIC BACKGROUND

BENZENE'S EFFECTS ON THE CHROMOSOME

Exposure to benzene can be toxic to the bone marrow and bone marrow–derived cells of humans and other species. In humans, a spectrum of conditions is induced that depends on the level and duration of exposure; these include pancytopenia, aplastic anemia, and acute myeloid leukemia (reviewed in Goldstein and Witz 2000). Studies have suggested that induction of chromosomal aberrations may play a role in benzene-induced carcinogenesis, and detecting these aberrations may serve as a marker of benzene's early effects. The genetic alterations induced include translocations, deletions, and aneuploidy (see sidebar). For example, workers occupationally exposed to benzene show an increased frequency of chromosomal aberrations in peripheral blood lymphocytes (Ding et al 1983; Aksoy 1988; Sasiadek 1992; Eastmond 1993); similar chromosome-damaging effects also have been shown in animals exposed to benzene (Tice et al 1980; Rithidech et al 1987; Ciranni et al 1991). It is noteworthy that the chromosomal aberrations described in workers exposed to benzene are also common characteristics of human leukemias, such as acute myeloid leukemia, and it is suspected that these aberrations may play a role in the induction of the disease (Kagan 1993; Hagenmeijer and Grosveld 1996).

ASSAYING CHROMOSOMAL ALTERATIONS BY THE FLUORESCENCE IN SITU HYBRIDIZATION TECHNIQUE

Earlier studies of benzene's effects on chromosomal structure used conventional cytogenetic techniques, which are laborious and prone to technical artifacts; in addition, data can be derived from only a small number of cells, generally 50 to 100. Thus, these techniques are of limited usefulness in population biomonitoring studies that involve many samples. Furthermore, those techniques can be used only for cells that are in metaphase, such as activated lymphocytes.

More recent studies have used FISH, a technique applied extensively in the chromosomal analysis of tumor cells (eg, Coleman et al 1997; Veldman et al 1997). Chromosomal alterations are detected by evaluating the in situ hybridization of specific fluorescent DNA sequences (probes) to regions of chromosomal DNA (Trask et al 1993). The brightly fluorescent spots at the hybridization site can be easily detected by fluorescence microscopy and the number of chromosomes in the nucleus can be determined by counting the number of regions of hybridization.

This approach offers the advantages of rapid detection of chromosomal alterations in large numbers of cells and in cells that are not dividing; that is, alterations may be detected in interphase as well as in metaphase nuclei. Estimating chromosomal alterations in interphase cells offers the possibility of studying effects in terminally differentiated cells such as polymorphonuclear leukocytes (PMN). Thus, chromosomal alterations induced by benzene in PMN can be compared with similar effects in activated lymphocytes, the cells most commonly used in cytogenetic studies.

When Eastmond applied for funding in 1993, relatively little was known about the precise nature of the chromosomal alterations induced by benzene exposure in humans or in other species. In his application, Eastmond referred to preliminary results obtained by Dr Martyn Smith and colleagues (University of California, Berkeley) in a study of Chinese workers occupationally exposed to benzene. Using a chromosome 9 probe in a FISH assay, Smith and colleagues detected hyperploidy in interphase lymphocytes from workers exposed to very high levels (90 ppm median concentration) of benzene (Zhang et al 1996). Because the chromosomal region targeted by this probe was highly prone to breaks, however, Eastmond considered the possibility that the number of abnormal cells reported in the occupationally exposed Chinese workers might not all have been due to aneuploidy. To address this issue, Eastmond proposed to use a new FISH method he had developed using two probes (the *tandem labeling* approach, described in more detail in the Study Design section below). Using this approach, he had previously demonstrated that concentrations around 100 μM of the benzene metabolite hydroquinone increased hyperploidy of chromosome 1 in interphase human lymphocytes and increased breakage within the 1q12 region of this chromosome

CYTOGENETIC TERMS

Chromosome Numbers

Ploidy refers to the number of sets of chromosomes within a cell or organism. Most cells are ***diploid*** because they contain two sets of chromosomes ("2n"), one from each parent (46 total chromosomes per cell in humans). Some cells are ***haploid*** ("n") because they contain only one set of chromosomes. Having more than two sets of chromosomes is known as ***polyploidy***. ***Aneuploidy*** indicates that the number of chromosomes in a cell is not an exact multiple of the haploid set for the species. ***Hypoploidy*** describes a cell or organism that has less than the normal number of chromosomes for the species, the opposite of ***hyperploidy***, in which the organism has more than the normal number of chromosomes.

Chromosome Structure

Chromatin refers to the complex of chromosomal DNA plus protein found in the cell's nucleus. Structurally, it can be divided into two broad categories: ***euchromatin***, gene-rich chromosomal regions that are diffuse and uncondensed during interphase (see below) and condensed at the time of nuclear division; and ***heterochromatin***, chromosomal regions with few genes that remain condensed during interphase and at the time of nuclear division.

The ***centromere*** is the short region of a chromosome that holds the DNA strands together while the cell divides (***mitosis***; see below). The centromere divides the human chromosome into two arms: the short or ***p arm*** and the long or ***q arm***. Each arm terminates in a ***telomere***, a repetitive sequence that prevents the end of one chromosome from fusing with the end of another.

The precise identification of genetic regions on a particular chromosome uses a convention that numbers the sequence of light and dark bands observed after staining the chromosome with particular dyes. For example, ***9q12*** identifies a region close to the centromere on the long (q) arm of chromosome 9.

Cell Cycle

Interphase is the main period in the cell cycle in which the cell is not undergoing mitosis. The G_0 stage is a quiescent stage within interphase in certain cells. ***Mitosis*** is the multistep process of cell division of which ***metaphase*** is the second step. The ***mitotic spindle*** is a microtubular structure that separates chromosomes during mitosis.

(Eastmond et al 1994). Hyperploidy of chromosome 9 also was seen in these cells. Studies by Smith and colleagues in the human myeloid cell line HL-60 also suggested that a benzene metabolite, in this case 1,2,4-benzenetriol, induced aneuploidy of chromosome 9 (Zhang et al 1994). As a consequence of these findings, Eastmond chose to examine abnormalities in human chromosomes 1 and 9 in the current study of benzene-exposed workers.

Eastmond also had used the FISH approach to characterize the origin of micronuclei formed in the cells of CD-1 mice after benzene exposure (Chen et al 1994). These studies suggested that the pattern of benzene-induced chromosomal damage might be different in different cell types, with chromosome breakage predominating in bone marrow erythrocytes and chromosome loss predominating in splenic lymphocytes. In the current study, Eastmond proposed to explore further whether benzene-induced chromosomal damage might be different in different cell types and to characterize the origin of chromosomal damage in bone marrow cells from $B6C3F_1$ mice exposed to benzene for different durations and at different levels.

EFFECT OF BENZENE METABOLITES ON TOPOISOMERASE II

The mechanism by which benzene induces carcinogenic effects is currently not known. Because benzene is rapidly metabolized along multiple pathways when it enters the body, it is likely that one or more benzene metabolites may be responsible for its toxic effects. Eastmond and others have tried to identify these metabolites (Greenlee et al 1981; Eastmond et al 1987; Goldstein 1989; Smith et al 1989).

In his application to HEI, Eastmond proposed a novel hypothesis to explain the mechanism of action of benzene metabolites on the chromosome. He suggested that the effects might not be direct, through interactions with DNA, but indirect, through interactions with enzymes or proteins involved in maintaining chromosomal structure. He described how some metabolites of benzene exhibit structural and functional similarities with a recently discovered class of leukemia-inducing agents known as epipodophyllotoxins (Pedersen-Bjergaard and Philip 1991). These compounds exert genotoxic and carcinogenic effects by inhibiting topoisomerase II, one member of a family of

Chromosomal Aberrations

A ***clastogen*** is an agent that causes chromosomal breaks. ***Deletion*** is a chromosome abnormality in which part of a single chromosome is lost. ***Translocation*** is when a segment of chromosome moves from one location to another either within the same or a different chromosome. A ***micronucleus*** is a small nucleus that has formed from chromosomal fragments or from entire chromosomes during mitosis and is separate from the main nucleus of a cell.

Monosomy is the state in a normally diploid cell or organism in which one or more chromosome pairs is represented by only one chromosome of the pair. ***Trisomy*** indicates the presence of an additional whole chromosome, and ***tetrasomy*** the presence of two extra copies of a chromosome.

FLUORESCENCE IN SITU HYBRIDIZATION TECHNOLOGY

A ***probe*** is a piece of DNA, usually specifically synthesized, that binds to (or ***hybridizes with***) a complementary sequence on one strand of chromosomal DNA. In the ***fluorescence in situ hybridization (FISH)*** technique, the probe is coupled with a fluorescent label; as a consequence, hybridization to the chromosome can be detected by fluorescence microscopy. In the ***tandem labeled FISH approach***, two different probes are used that bind to adjacent regions of a specific chromosome; each probe is coupled to a different fluorescent label. The detection of chromosomal aberrations using this approach is schematically illustrated in Figures 2, 3, and 21 of the Investigators' Report.

In many of the FISH analyses, the investigators used probes specific for ***satellite DNA***, sections of repetitive DNA sequences in the centromeric regions of all chromosomes. The regions Dr Eastmond studied include the ***mouse major (M) satellite***, located in the centromeric heterochromatin adjacent to the long (q) arm of the mouse chromosome, and the ***mouse minor (m) satellite***, which encompasses the centromeric region and is linked to the telomere of the short arm. The human ***α-satellite*** sequences targeted by Dr Eastmond and colleagues encompass the centromeres of chromosomes 1 and 9. The targeted ***classical-satellite*** regions (1q12 and 9q12) are located very close to the centromeric α-satellite sequences on the long (q) arms of chromosomes 1 and 9.

chromosomal enzymes that participate in a range of cellular processes including DNA replication and transcription, chromosomal segregation and DNA repair, and the maintenance of genomic stability. Eastmond postulated that benzene metabolites that have structural features similar to the epipodophyllotoxins also might affect the function of topoisomerase II, and that inhibiting topoisomerase II activity at critical stages of the cell cycle might lead to chromosome breakage, aneuploidy, or cell death. He proposed to test these concepts in the current study.

TECHNICAL EVALUATION

AIMS AND ATTAINMENT OF STUDY OBJECTIVES

The objectives of Eastmond's research were to utilize the FISH technique to characterize the nature and persistence of chromosomal alterations induced by benzene in mice and humans, and to determine the role of topoisomerase II inhibition in benzene-induced chromosomal changes. The specific aims were:

1. to characterize the origin of chromosomal alterations that occur in mice after short-term and longer-term benzene exposure;
2. to determine the role of topoisomerase II inhibition in the induction of chromosomal alterations induced by benzene; and
3. to characterize chromosomal alterations seen in populations of workers with current and previous exposure to various levels of benzene.

STUDY DESIGN

Animal Exposure to Benzene

The investigators administered benzene by oral gavage to male $B6C3F_1$ mice once per day, 5 days/week (followed by two days without dosing) for approximately 2, 6, or 12 weeks. For the 2-week study, 6 mice received 8 doses of 50, 100, or 400 mg/kg over a 10-day period. For the 6- and 12-week studies, mice received 100 or 400 mg/kg (4 mice receiving 29 doses over 40 days in the 6-week study, and 10 mice receiving 59 doses over 81 days in the 12-week study). Bone marrow cells from these animals were analyzed for chromosomal aberrations 24 hours after the final dose of benzene.

Human Occupational Exposure to Benzene

Eastmond and colleagues obtained slides of peripheral blood cell samples—blood smears and lymphocytes stimulated in vitro—from two groups of investigators who are studying the effects of benzene in occupationally exposed workers. The first set was from benzene-exposed workers and unexposed control subjects in Estonia and were prepared by Drs Angelo Carere and Riccardo Crebelli at the Italian Institute of Health in Rome. Eastmond obtained samples from a subset of the study group: 17 factory workers at a shale oil petrochemical plant in Kohtla-Järve, Estonia. Of these, 12 worked in benzene production and 5 in the coke oven operation (and were exposed to lower levels of benzene); 8 unexposed control subjects from a rural village were also included. Table 17 of the Investigators' Report indicates that the mean ($\pm$ SD) levels of exposure in the benzene-exposed groups were 4.1 ± 8.0 mg/m^3 (equivalent to 1.3 ppm; 8-hour time-weighted average) for the workers in benzene production and 1.1 ± 0.5 mg/m^3 (0.3 ppm; 8-hour time-weighted average) for the workers in the coke oven operation. Table 17 also indicates that levels of blood benzene, urinary *trans,trans*-muconic acid, and *S*-phenylmercapturic acid appeared to be higher in benzene production workers than in coke oven workers, which were, in turn, somewhat higher than the levels in control subjects. These compounds were used in Dr Carere's study as biomarkers of benzene exposure. A full characterization of the workers and their levels of exposure to benzene in this multicenter study are reported by Kivisto and associates (1997).

The second set of slides was derived from benzene-exposed workers and control subjects in Shanghai, China. The individuals from whom the samples were obtained formed part of a group studied by Drs Martyn Smith and Nathaniel Rothman in a joint study between the US National Cancer Institute and the Chinese Academy of Preventive Medicine (Rothman et al 1996). Slides were prepared from three groups:

- 44 workers currently exposed to benzene; median exposure concentration was 31 ppm as an 8-hour time-weighted average (range 1.6 to 328.5 ppm). For cytogenetic analysis, this currently exposed group was split into subgroups of < 31 and ≥ 31 ppm exposure.
- 50 workers who had previously experienced benzene poisoning that had resulted in myelotoxicity. As a result of this earlier exposure to high levels of benzene, these workers had been removed from their jobs in the factory. Whether members of this subgroup had been subsequently exposed to benzene in the factory is not clear. According to the Investigators' Report, ". . . for the most part, these workers had been removed from further exposure to benzene several years earlier."

- 44 control individuals who worked in a sewing machine manufacturing facility and an administrative facility; these subjects were matched by age and gender with the workers currently exposed to benzene.

Key characteristics of the currently exposed workers and control subjects are presented in Tables 18 and 19 of the Investigators' Report. Levels of the urinary benzene metabolites phenol and *t,t*-muconic acid (the biomarkers of benzene exposure used in the study) were much greater in the currently exposed workers than the control group (5-fold for phenol and 130-fold for *t,t*-muconic acid).

Analysis of Chromosomal Alterations

The investigators used three distinct approaches.

A standard cytogenetic assay for detecting micronuclei in nucleated erythrocytes from mouse bone marrow Cells from benzene-exposed and control animals were stained with acridine orange and scored (for each animal) by fluorescence microscopy (reviewed in MacGregor et al 1987; Mavournin et al 1990). Newly synthesized polychromatic erythrocytes (PCEs), which contain RNA, stained orange-red; more mature normochromatic erythrocytes (NCEs), which lack RNA, did not stain with the dye.

Tandem labeled FISH for mouse cells using non–chromosome-specific probes This assay was used to characterize more precisely the origin of micronuclei that developed in red blood cells from mouse bone marrow after benzene exposure. After fixing the cells with methanol and paraformaldehyde, the investigators evaluated the hybridization of two DNA probes labeled with different fluorescent reagents to adjacent (that is, "tandem") stretches of chromosomal DNA. They used fluorescent probes specific for the large centromeric regions known as the major (M) and minor (m) "satellite" regions (see sidebar), which are found on all mouse chromosomes except the Y-chromosome.

Eastmond and colleagues interpreted the fluorescence patterns they detected as follows:

- Hybridization regions for both major and minor satellites indicated the likely presence of the entire chromosome within the nucleus and, thus, chromosome loss as the origin of the micronucleus (referred to as M+m+).
- A hybridization region for the major, but not the minor, satellite sequence indicated a break within the major satellite DNA, that is, within the mouse centromeric heterochromatin (referred to as M+m−).
- No hybridization signal indicated a break outside the major and minor satellite DNA regions, that is, a break within the euchromatin (referred to as M−m−).

FISH using chromosome-specific DNA probes To evaluate benzene-induced changes in specific mouse chromosomes, Eastmond and colleagues used a single-color FISH approach to analyze effects in mononuclear and polymorphonuclear cells from bone marrow. They used fluorescent probes specific for subcentromeric regions of mouse chromosomes 8 and 14. These two chromosomes were chosen because, at the time of the study, these were the only chromosomes for which adequate probes were available.

For the evaluation of human cells, the investigators used tandem fluorescent probes specific for satellite DNA in the centromeric and pericentromeric regions of chromosomes 1 and 9, that is, 1cen and 1q12, and 9cen and 9q12. As described in the Scientific Background section, Eastmond's earlier findings and data from Smith and colleagues, including Smith's preliminary findings from the US National Cancer Institute study in China, indicated that these chromosomes were expected to be affected by benzene (Eastmond et al 1994; Zhang et al 1994, 1996). Eastmond and associates used a red fluorescent probe that targets classical-satellite DNA located in the pericentromeric heterochromatin and a yellow-green fluorescent probe that targets α-satellite DNA specific for the adjacent centromeric region. They interpreted the hybridization patterns as follows:

- A red fluorescent spot adjacent to a yellow spot indicated an intact chromosome.
- Three hybridization regions in which two had adjacent red and yellow fluorescence and a third showed only red fluorescence was scored as a cell with two copies of either chromosome 1 or chromosome 9 with a breakage event having occurred within the chromosomal region targeted by the classical-satellite probe.
- A wide separation between red and yellow spots was scored as breakage between the hybridization regions targeted by the DNA probes.
- Hybridization regions appearing as doublets or diffused signals were scored as one hybridization region.

In the Estonian worker study, tandem labeled probes for both chromosomes 1 and 9 were evaluated in slides made from blood smears (containing mononuclear cells and PMN) and from lymphocytes cultured for 48 hours with the polyclonal activator (or "mitogen") phytohemagglutinin (PHA) to stimulate cell division. In the Chinese worker study, tandem labeled probes specific for chromosome 1 were evaluated in slides of lymphocytes cultured for 72 hours with PHA. The investigators were unable to obtain FISH data from blood smears prepared from subjects in the Chinese study; as described further in the Discussion section, the investigators believe that the conditions under

which these slides were prepared were not optimal for hybridization of the FISH probe.

All the FISH analyses were carried out by Eastmond and colleagues, apart from the chromosome 1 analysis of slides prepared from the cultured lymphocytes of Estonian workers. This analysis was performed in Rome by Dr Crebelli and associates. For each subject, 1,000 cells were counted on slides of cultured lymphocytes, and 500 cells counted on slides of blood smears (containing PMN and mononuclear cells).

Effects of Benzene and Benzene Metabolites on Topoisomerase II Activity

The investigators tested whether (1) in vitro treatment with a number of benzene metabolites or putative metabolites, or (2) in vivo exposure to benzene would inhibit the activity of topoisomerase II. Enzyme activity was assayed by monitoring the generation of either open or relaxed circular DNA monomers from intertwined (or "catenated") DNA rings (Marini et al 1980). The ability of a metabolite to act as a topoisomerase II inhibitor was tested by assessing whether, as detected by gel electrophoresis, it decreased or prevented the formation of such decatenated DNA structures.

In vitro inhibition of topoisomerase II activity was initially tested using purified human enzyme. Because some benzene metabolites are generated by oxidation processes (see Figure 1 in the Investigators' Report), Eastmond and colleagues evaluated whether benzene metabolites needed to be bioactivated in vitro by an oxidation pathway to inhibit topoisomerase II activity. To test this, they incubated benzene metabolites at a range of concentrations with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) at room temperature before adding the incubation mix to the topoisomerase II assay. In an attempt to optimize assay conditions, the investigators tested a range of HRP and H_2O_2 concentrations (from 0.07 to 0.25 U/mL for HRP and 55 to 500 μM for H_2O_2) and incubation times (5 minutes to 1 hour).

The ability of benzene metabolites, in the presence or absence of bioactivation, to inhibit topoisomerase II activity in the nuclear extracts of the human myeloid cell line HL-60 was tested by incubating the compounds with the cells for up to 48 hours. The effects of benzene on topoisomerase II activity in vivo were tested in mice administered 440 mg/kg benzene by gavage for 3 consecutive days. Topoisomerase II activity was assayed in erythrocyte-free, nucleated bone marrow cells 24 hours after the final benzene treatment.

KEY RESULTS

CHROMOSOMAL ALTERATIONS IN BENZENE-EXPOSED MICE

- ***Increased concentration and duration of benzene exposure increased the frequency of micronuclei in polychromatic erythrocytes from bone marrow.*** A dose-related increase in the frequency of micronuclei was seen at each time point; that is, at 2, 6, and 12 weeks of exposure, 400 mg/kg benzene orally administered daily induced higher numbers of micronuclei than did 100 mg/kg benzene. In addition, greater numbers of micronuclei were detected after 12 weeks of exposure than after 2 or 6 weeks of exposure at each dose.
- ***Fluorescence in situ hybridization analyses of red blood cells from bone marrow indicated that benzene's effects were attributable predominantly to chromosome breakage and also to chromosome loss.*** Using the major and minor satellite FISH probes, the benzene-induced increases in erythrocyte micronucleus frequencies (described in the last paragraph) were found to be due primarily to chromosome breakage, particularly within euchromatin (M−m− pattern), although increases in chromosome loss also were seen (M+m+ pattern).
- ***Fluorescence in situ hybridization analyses of mononuclear and polymorphonuclear cells from bone marrow suggested a small benzene-induced effect on hyperdiploidy.*** Using the probes specific for chromosomes 8 and 14 in nucleated cells from bone marrow, little or no effect of benzene on hyperdiploidy was noted at any single time point. When the results for the two probes and two cell types were combined, however, a small dose-related increase in the frequency of hyperdiploidy was observed at all exposure durations (2, 6, and 12 weeks).

CHROMOSOMAL ALTERATIONS IN BENZENE-EXPOSED WORKERS

Estonian Study: Workers Currently Exposed to Benzene

- ***In blood smears, no differences in hyperdiploidy or in chromosomal breakage in the 1cen-1q12 region were found between exposed workers and control subjects.*** Tandem labeled FISH analyses of PMN and unstimulated lymphocytes in blood smears indicated no statistically significant differences in chromosomal alterations among benzene factory workers,

coke oven operation workers (who were exposed to lower benzene levels), and control subjects. The frequency of breakage in the labeled region of chromosome 1 was generally higher in PMN than in unstimulated lymphocytes.

- ***In cultured lymphocytes, small increases in hyperdiploidy and in chromosomal breakage in 1cen-1q12 and 9cen-9q12 regions were detected in benzene factory workers compared with control subjects.*** Tandem labeled FISH analysis of chromosomes 1 and 9 showed a modest increase in chromosomal breakage in the cells from benzene production workers compared both with coke oven operation workers and unexposed control subjects. The median frequencies of breakage in the 1cen-1q12 region were 0.2% in control subjects, 0.4% in coke oven workers, and 0.6% in benzene factory workers. The difference between the frequencies in the benzene factory workers and control subjects was statistically significant. The frequency of breakage in the 9cen-9q12 region was higher than that observed in the 1cen-1q12 region: 0.6% in control subjects, 0.7% in coke oven workers, and 1.0% in benzene factory workers. Differences for chromosome 9 breakage among the three groups did not attain statistical significance ($P = 0.053$; Kruskall-Wallis test). Using the Mann-Whitney U test, however, a statistically significant excess of 9cen-9q12 breaks was observed in comparing the incidence in benzene-exposed workers with that in the control group ($P < 0.05$). The investigators' analysis of individual results revealed a strong correlation between the results obtained with the two chromosomes.

  The incidence of hyperploidy in both chromosomes was slightly higher in the benzene factory workers than in the other groups (see Figure 23). The differences did not attain statistical significance for either chromosome, possibly reflecting the small sample sizes and the overall low frequency of hyperploidy observed.

  No correlation was seen between the frequency of breakage in either the 1q12 or 9q12 region and exposure biomarkers, age, or smoking status. In addition, no association was seen between hyperdiploidy and the exposure biomarkers.

Chinese Study: Workers Currently Exposed to Benzene

- ***No excess in chromosome 1 aberrations was detected.*** Using tandem labeled FISH with probes for chromosome 1 on cultured lymphocytes in interphase, no significant differences were seen for either breakage or hyperdiploidy between workers and control subjects. Similar results were obtained when the workers were divided into high-exposure ($\geq$ 31 ppm) and low-exposure ($<$ 31 ppm) subgroups. The data suggested, however, that workers with the higher exposure had a slight increase in hyperdiploid cells.
- ***Associations were found between chromosome alterations and biomarkers of benzene exposure.*** The investigators found significant correlations between hyperdiploidy for chromosome 1 and concentrations of both urinary phenol and *t,t*-muconic acid in the exposed workers (see Figure 26). They did not find an association, however, between chromosome breakage and levels of either urinary phenol or *t,t*-muconic acid.

  Likewise, no significant associations were observed between the frequency of breakage or hyperdiploidy and age, smoking status, or cigarettes per day. However, a weak but statistically significant difference in the frequency of breakage was observed between males and females ($P = 0.038$; t test): The mean ($\pm$ SD) frequency of breakage in cells from men (1.485‰ $\pm$ 0.821‰) was higher than that seen in cells from women (1.116‰ $\pm$ 0.789‰).

Chinese Study: Workers Previously Poisoned by Benzene

- ***No excess in chromosome 1 aberrations was detected.*** Using FISH with the tandem probes for chromosome 1 on cultured lymphocytes, the investigators found no increased frequency of breakage affecting the 1cen-1q12 region nor of hyperdiploidy for chromosome 1 in the previously poisoned workers compared with control subjects (see Figure 27). No significant associations were detected between the frequency of breakage or hyperdiploidy for cigarettes per day, pack years, gender, current benzene exposure, alcoholic drinks per week, or historical benzene exposure levels. However, a significant positive association between frequency of breakage and age was observed.

Inhibition of Topoisomerase II

- ***Inhibition of purified human topoisomerase II was specific to each metabolite and generally enhanced by incubating the metabolite with horseradish peroxidase and hydrogen peroxide.*** Some benzene metabolites, such as *t,t*-muconic acid (100 μM) and 1,4-benzoquinone (10 μM), completely inhibited topoisomerase II activity when added directly to the

purified enzyme. Other metabolites, including phenol, 2,2′-biphenol, and 4,4′-biphenol, had no inhibitory effect on the enzyme even at the highest concentrations tested (500 μM), but were inhibitory at 10 to 100 μM after in vitro bioactivation in the presence of HRP and H_2O_2. Catechol, hydroquinone, and 1,2,4-benzenetriol inhibited topoisomerase II activity in both the presence and absence of bioactivation, but inhibition was achieved at much lower concentrations after bioactivation (around 10 μM, compared with 250 to 1,000 μM in the absence of bioactivation).

- ***Some benzene metabolites inhibited topoisomerase II activity in the human myeloid cell line HL-60.*** Adding 100 μM 1,2,4-benzenetriol to human HL-60 cells for 2 hours decreased topoisomerase II activity by approximately 50%. Adding 500 μM 4,4′-biphenol or 10 μM hydroquinone with H_2O_2 (with the intent of increasing the cells' ability to bioactivate the metabolites) also inhibited HL-60 topoisomerase II activity by the same amount. Catechol (500 μM) plus H_2O_2 did not inhibit the cells' topoisomerase II activity. Under conditions in which 4,4′-biphenol and hydroquinone inhibited topoisomerase II activity in HL-60 cells, lower levels of the enzyme were recovered from treated cells than from untreated control cells. The investigators also tested topoisomerase II activity in different dilutions of nuclear extracts prepared from HL-60 cells both untreated and treated with benzene metabolites; they did not find a linear relation between the extract concentration and enzyme activity in either treated or untreated cells.
- ***Administering benzene to mice inhibited topoisomerase II activity.*** In three experiments, topoisomerase II activity was decreased by approximately 40% in nucleated cells from bone marrow 24 hours after the final administration of benzene.

DISCUSSION

BENZENE-INDUCED CHROMOSOMAL ABERRATIONS IN MICE AND HUMANS

Interpretation of Results from Fluorescence in Situ Hybridization

Using conventional cytogenetic approaches, Eastmond and colleagues detected increases in micronuclei in nucleated erythrocytes from the bone marrow of $B6C3F_1$ mice; the increases were dependent on both the dose and exposure duration of benzene, and were seen predominantly in the newly synthesized subset of bone marrow erythrocytes (PCEs). These results confirm and extend Eastmond and colleagues' previous findings in CD-1 mice (Chen et al 1994). The dose- and duration-dependent increases in micronuclei detected in the current study suggest that benzene has a cumulative effect on the induction of chromosomal aberrations in mice. The mechanism by which benzene might exert such cumulative effects, and particularly in recently synthesized erythrocytes, is not clear; Eastmond and colleagues speculate that this may be the result of (1) changes in the profile of benzene metabolites with increasing dose and duration or (2) increasing genomic instability.

Using the tandem labeled FISH technique to characterize the benzene-induced chromosomal alterations in bone marrow cells, the investigators detected predominantly chromosome breakage (with some chromosome loss) in erythrocytes and a small increase in hyperdiploidy in leukocytes. These findings suggest that benzene induces different types of chromosomal aberrations that can be seen in different cell lineages. This may result from different pathways of benzene metabolism and the predominance of distinct metabolites in different cell populations. Alternatively, the observed differences in chromosomal alterations between leukocytes and erythrocytes may be attributable to differences in the ability to detect aberrations in these distinct cell types.

In cells from humans occupationally exposed to benzene, the investigators also assessed chromosomal changes by tandem labeled FISH in lymphocytes stimulated to proliferate in vitro. The total number of chromosomal changes detected in the human study, however, was not large and the results were not as clear cut as those obtained in the mouse study described above.

The differences between the results in humans and in mice may be attributable to differences in levels of benzene exposure, but this is not clear. Comparisons of exposure across species are always difficult, and particularly so in the current study in which mice were exposed to multiple doses (50, 100 or 400 mg/kg) via gavage and humans were exposed via continuous workplace inhalation. On the basis of a calculation of human ventilation rate in the Chinese study, a median human exposure to benzene of 31 ppm over 8 hours would correspond to around 1,200 mg benzene; for a 70-kg individual, this would work out to inhaling approximately 17 mg/kg every day the individual was at the factory.

The results in the human populations were the opposite of those expected; that is, more chromosomal alterations were detected in the lymphocytes of the small group of Estonian workers exposed to low levels of benzene (1 ppm

mean) than in the lymphocytes from the large group of Chinese workers exposed to much higher levels (31 ppm median). Furthermore, workers in China who had been previously poisoned as a result of exposure to very high levels of benzene did not show chromosomal alterations in the 1cen1q12 region assessed.

These unexpected findings illustrate the potential difficulties of applying the FISH approach to large human genetic toxicologic studies that involve assays performed at different locations. As the investigators pointed out, the patterns detected by FISH are critically dependent on the conditions under which the probes hybridize to the cells' chromosomes. For example, factors such as the purity of methanol and acetic acid used as fixatives for slides, the level of humidity when preparing slides, and the time and conditions of storage may all influence the hybridization efficiency of the probes and thus the hybridization patterns detected. For these reasons, the investigators believe, they were unable to obtain usable data from the blood smears from workers in the Chinese study.

In addition, scoring the hybridization patterns is dependent on the observer and different laboratories may apply different scoring criteria. This may partly explain why levels of hyperdiploidy noted in the control values of the Estonian worker study were very low compared with the investigators' previous studies (Eastmond et al 1995; Rupa et al 1995). In the current study, control values in the Estonian groups were also somewhat lower than those seen in the Chinese workers. Interestingly, the control frequencies reported by Zhang and coworkers (1996) in the NCI study of the Chinese workers are considerably higher, with mean hyperdiploid frequencies of 0.7%. The reasons for these differences in control frequencies are not clear.

Similarly, Eastmond and colleagues detected an increase in the frequency of cells that exhibited zero and one hybridization region in the bone marrow cells of the benzene-treated animals at one or two time points. Because this pattern was highly dependent on hybridization conditions and was variable in untreated cells, the investigators were not sure if the decrease in hybridization signals represented a true change in the number of chromosomes. As a result of this combination of factors, the investigators placed considerably less weight on the hypodiploidy endpoint compared with the endpoints that measured micronuclei or hyperdiploidy.

For these reasons, the results of studies performed in one laboratory may differ from those obtained in another even if they are studying the same chromosome. As a result, caution must be used when comparing FISH results obtained from different laboratories using different protocols even in the same study as well as when comparing FISH results from different studies. These points also illustrate the potential limitations in using the FISH approach to identify chromosomal aberrations as biomarkers of benzene's effects in large multicenter studies.

The structure of the chromosomal region to which the FISH probe binds is also critical in determining the extent of hybridization. For example, Eastmond and colleagues noted that the FISH hybridization patterns using mouse chromosome 8 and 14 probes were somewhat diffuse because the chromosomal regions targeted by the probes comprised sequences of interspersed repeats. The investigators thought that the technique was satisfactory for investigating cells in interphase and for detecting increases in chromosome number (hyperdiploidy and polyploidy), but not reliable for detecting chromosome loss (Eastmond and Pinkel 1990; Eastmond et al 1995). Thus, the FISH approach may not reliably detect all types of chromosomal aberrations.

Comparison of Results from Estonian and Chinese Worker Studies

In addition to the technical issues described above, one must consider several other possible explanations for the findings that chromosomal aberrations were detected in the Estonian workers exposed to low levels of benzene but not in the more highly exposed Chinese workers. First, the positive findings in Estonian workers may have resulted from random fluctuations in data. The investigators argue convincingly that this was unlikely because increases in chromosome breaks in the Estonian workers were detected by using probes specific for different chromosomes and were tested in two different laboratories (one in Italy, one in California). If the differences in numbers of chromosomal aberrations between Estonian and Chinese workers were not artifactual, they could also have resulted from a number of other factors. These include an agent or agents distinct from benzene in the Estonian work environment (such as polynuclear aromatic hydrocarbons), a difference in lifestyle factors (such as diet or medications) between the two populations, or an unusual dose-response curve in which lower benzene doses would induce higher numbers of aberrations. An additional explanation is that the expression of enzyme polymorphisms that affect the formation of chromosome-damaging benzene metabolites is different in these two occupational cohorts.

One further possibility to explain why chromosome breakage was detected in cells from Estonian workers but not in cells from Chinese workers was that peripheral blood lymphocytes from the two groups were stimulated with PHA for different durations of time. Lymphocytes from Estonian workers were harvested at 48 hours, a time

that the investigators say is optimal for detecting structural alterations. In contrast, lymphocytes from Chinese workers were harvested at 72 hours, when most of the cells should have been in their second metaphase. This time point is preferable for detecting aneuploidy, but not for detecting breaks: chromosome fragments can be lost when the cell cycles from the first to the second mitosis (Carrano and Natarajan 1988).

Comparison of Eastmond's Findings in the Chinese Worker Study with Those of Others

Smith and colleagues also evaluated chromosome structural and numerical alterations in the same set of benzene-exposed workers and control subjects (Zhang et al 1998, 1999). Using a single probe FISH approach, these investigators found that the benzene-exposed worker group showed an increase in hyperploidy and chromosome deletion for chromosomes 5 and 7 in one study (Zhang et al 1998), and an increase in hyperploidy for chromosomes 7 and 8 in another (Zhang et al 1999). Thus, the reported response was somewhat more definitive than that described by Eastmond and colleagues and suggests that benzene induces cytogenetic alterations in exposed humans. Comparing Smith's studies with Eastmond's studies is difficult, however, because different methods and probes were used and different chromosomes, which may be differentially sensitive to benzene's effects, were analyzed.

Exposure to benzene has been associated with the induction of leukemia, so the increase in chromosomal alterations in lymphocytes described by Smith and colleagues could be interpreted as indicating the potential for an increase in leukemia in the exposed group, although not necessarily for any individual in the group. Remember, however, that Smith measured cytogenetic effects in specific chromosomes, which he selected because such alterations in those chromosomes have been associated with leukemia (Smith and Zhang 1998). He did not report, and it is not currently known, if aneuploidy increases for other chromosomes that are not reported to be associated with leukemia, or if similar aneuploidies might be observed in other occupationally exposed groups not exposed to benzene, or even if certain aneuploidies increase with age.

In the future, developing a clearer understanding of how a specific chemical exposure induces a particular tumor should allow the selection of more specific genetic markers of response. The ability to use biomarkers in the future to predict cancer incidence will likely also require information on responses in specific target tissues.

Eastmond, like Smith, found that the frequency of a chromosomal aberration in the currently exposed Chinese workers correlated with the level of a urinary benzene metabolite: Eastmond found that the frequency of hyperdiploidy in chromosome 1 did not correlate with air levels of benzene, but did correlate with levels of the urinary benzene metabolites phenol and *t,t*-muconic acid). Likewise, Smith and colleagues found that hyperdiploidy of chromosome 9 correlated with urinary phenol (Zhang et al 1996). These findings suggest that the levels of these metabolites as biomarkers of internal dose may be more useful surrogates of benzene's effects than are measures of benzene concentration in the ambient air of the workplace. This may be significant for future benzene biomonitoring studies. The utility of several benzene biomarkers for monitoring occupational exposure to benzene is currently being explored in an HEI-funded study by Dr Qingshang Qu, New York University School of Medicine, in which Eastmond and colleagues are participating.

MECHANISM OF BENZENE'S ACTION THROUGH TOPOISOMERASE II

Eastmond's finding that a number of benzene metabolites inhibit topoisomerase II activity in vitro suggests that this may be a possible mechanism for the toxic effects of benzene on the chromosome. As the investigators discuss, the toxic effects could also occur through an event such as poisoning of the mitotic spindle and, as a consequence, benzene could induce a variety of chromosomal alterations including breakage and aneuploidy. The finding that many benzene metabolites must be activated in vitro with a peroxidase and H_2O_2 suggests that benzene bioactivation to one or more metabolites may be required to inhibit topoisomerase II.

The investigators found that administering benzene to mice lowered the levels of bone marrow–derived topoisomerase II, which indicates that benzene can affect topoisomerase II in vivo. It is not clear, however, whether this in vivo inhibition by benzene operates through the bioactivation mechanism involving a peroxidase and H_2O_2 that was used in the in vitro studies. After administering benzene, H_2O_2 may be produced by bone marrow cells in vivo as a consequence of generating reactive oxygen species. Bone marrow cells do not contain HRP, but some do express another peroxidase, myeloperoxidase. This enzyme is not found, however, in nucleated red blood cells, the cells in which benzene-induced chromosomal alterations were detected. Thus, this bioactivation mechanism remains an interesting but speculative way to explain benzene's effects in vivo.

In conclusion, inhibition of topoisomerase II activity by benzene metabolites is a plausible, but not necessarily the sole, mechanism by which benzene exerts its toxic or carcinogenic effects on chromosomal structure. Because the

investigators' results showed that in vitro topoisomerase II activity was not linear over a range of dilutions of HL-60 cell nuclei, it is not clear whether this assay can be used as a biomarker of early benzene effects. Further studies are required to evaluate the usefulness of this assay.

CONCLUSIONS

Eastmond and colleagues achieved several important goals in their study. They demonstrated that they could detect some types of benzene-induced chromosomal alterations in mice and humans using single and tandem labeled fluorescent probes. Controlled exposure studies in mice suggested dose- and time-dependent benzene-induced increases in chromosomal alterations, whereas the results of human biomonitoring studies were not as clearcut: Chromosomal alterations in a population of Chinese workers highly exposed to benzene did not differ from control levels; but a smaller group of Estonian workers exposed to lower levels of benzene showed chromosomal changes. Although the results indicate the feasibility of the approach, they also underline important limitations in the use of the tandem labeled FISH assay in large human studies.

These investigators were the first to show that benzene administration in vivo and some benzene metabolites or potential metabolites in vitro can inhibit the nuclear enzyme topoisomerase II. These findings suggest, but do not prove, that topoisomerase II may be an important target for benzene. Because the results of the topoisomerase II activity assay in vitro were not linear in the dilution range tested, however, topoisomerase II activity cannot be used at present as an indicator of early benzene effects.

In conclusion, the investigators were able to conduct initial tests of new biomarkers of benzene exposure and effects. Additional studies will help to establish whether using FISH with tandem probes or measuring topoisomerase II activity will be useful biomarkers for assessing ambient or occupational exposures to benzene.

ACKNOWLEDGMENTS

The Health Review Committee thanks the ad hoc reviewers for their help in evaluating the scientific merit of the Investigators' Report. The Committee is grateful to Dr Debra Kaden for her scientific oversight of the study, Dr Geoffrey Sunshine for his assistance in preparing its Commentary, and to John Abbott, Julia Campeti, Sally Edwards, Virgi Hepner, and Hope Steele for their roles in publishing this Research Report.

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Number 103
June 2001