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Validation and Evaluation of Biomarkers in Workers Exposed to Benzene in China

Qingshan Qu, Roy Shore, Guilan Li, Ximei Jin, Lung Chi Chen, Beverly Cohen, Assieh A Melikian, David Eastmond, Stephen Rappaport, Heyi Li, Doppalapudi Rupa, Suramya Waidyanatha, Songnian Yin, Huifang Yan, Min Meng, Witold Winnik, Eric SC Kwok, Yuying Li, Ruidong Mu, Bohong Xu, Xiaoling Zhang, and Keqi Li

Includes a Commentary by the Institute's Health Review Committee

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HEI STATEMENT
Synopsis of Research Report 115

Validation of Biomarkers in Workers Exposed to Benzene

Human exposure to benzene is widespread because it is a component of gasoline and is also used extensively as an industrial solvent. Exposure to high levels of benzene is associated with development of leukemia and other blood disorders, but the risks of exposure to low levels of benzene are not well understood. In the 1990s the Health Effects Institute initiated a research program designed to study the effects of exposure to toxic air pollutants at ambient levels. As one part of this research program, HEI's Request for Applications (RFA) 93-1 supported studies to develop reliable and sensitive assays for biomarkers of benzene exposure—both recent and longer-term—and of benzene effect. The biomarkers of *recent* exposure were urinary metabolites (measuring responses up to hours after exposure) and adducts of blood proteins (days to weeks after exposure). The biomarkers of *longer-term* exposure were chromosomal changes, integrating exposure over months to years. Because chromosomal changes may be determinants of subsequent health effects, they may also be considered early biomarkers of benzene effect. Chromosomal changes may also be due to causes other than exposure to benzene.

To validate the biomarkers characterized in these studies, another part of the research program, Request for Qualifications (RFQ) 95-3, "Transitional Epidemiology Studies for Benzene or 1,3-Butadiene Biomarkers," solicited applications from investigators with access to suitable human populations exposed to benzene or butadiene. HEI funded a study by Dr Qingshan Qu of New York University School of Medicine to evaluate putative biomarkers in workers occupationally exposed to benzene in China.

APPROACH

Qu and colleagues recruited 181 healthy workers in several factories in the Tianjin region of China. These subjects formed part of a cohort of thousands identified by the US National Cancer Institute (NCI) and the China Academy of Preventive Medicine for a study to evaluate tumor incidence in benzene-exposed workers (NCI/China study). In phase 1 of their study, Qu and colleagues evaluated the suitability of using urinary metabolites, blood adducts, or chromosomal aberrations in polymorphonuclear leukocytes and lymphocytes as benzene biomarkers in 25 heavily exposed and 25 unexposed workers. The urinary metabolites measured were phenol, catechol, hydroquinone, benzene triol, *S*-phenylmercapturic acid (*S*-PMA), and *trans,trans*-muconic acid (*t,t*-MA). The blood adducts measured were benzene oxide and benzoquinone adducts of albumin.

In phase 2, the investigators used biomarkers validated in phase 1 of the study to evaluate relations between benzene exposures and levels of these biomarkers in another 105 benzene-exposed workers and 26 unexposed workers. The investigators focused on obtaining samples from workers whose current-day exposures to benzene were no more than 5 ppm, representing the low end of occupational exposure. Qu and colleagues also evaluated whether the number and type of blood cells decreased in the exposed subjects because such decreases may be early indicators of a response to occupational benzene exposure. Some biological samples were analyzed in China and some in the United States.

RESULTS AND INTERPRETATION

This study has made important contributions regarding the utility of biomarkers of benzene exposure in occupational settings. It is the first to evaluate multiple possible biomarkers of benzene across a wide range of exposures and to show effects at the lowest end of the range. In addition to using sensitive assays for urinary metabolites and blood adducts, Qu and colleagues made great efforts to accurately measure and monitor personal exposures to a wide range of benzene levels in the workplace—critical features for assessing the accuracy of biomarker information. The investigators also paid careful attention to quality control issues.

The study's most novel finding was that benzene exposure was associated with decreases in the numbers of circulating neutrophils and, to a lesser

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and directed by Dr Qingshan Qu at the New York University School of Medicine, Tuxedo NY. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

extent, lymphocytes. The decrease in neutrophil numbers is interesting because long-term human exposure to high levels of benzene has been previously associated with the development of cancer in bone marrow precursor cells that give rise to neutrophils. This result—indicating that changes in neutrophil numbers may be a sensitive marker of benzene effects—needs to be corroborated, however, because other studies have found changes in lymphocyte, but not neutrophil, numbers.

A key positive feature of the study design was Qu's 2-step approach to validating possible biomarkers in phase 1 before proceeding to the larger study in phase 2. The phase 1 results indicated that *S*-PMA and *t,t*-MA, minor metabolites of benzene found in urine, might be the most useful markers of recent benzene exposure. Combined analysis of phase 1 and 2 results confirmed the suitability of *S*-PMA and *t,t*-MA as biomarkers for this purpose: both markers had low background levels in unexposed workers and increased levels in exposed workers. *S*-PMA was found to be the most useful biomarker for recent exposure to benzene because of the extent of the change in its level, its sensitivity in correlating with low occupational benzene exposures, and its specificity for benzene exposure. The urinary metabolites phenol, hydroquinone, and catechol were less sensitive to changes in benzene exposure and had higher background levels than *S*-PMA and *t,t*-MA. Therefore, these markers were less suitable for detecting dose-dependent variation across the spectrum of benzene exposures. Benzene triol was found to be unsuitable as a biomarker.

Exposure-dependent changes in blood adduct levels (half-life in blood of approximately 14 days) were found to be suitable measures for evaluating recent exposure although the background levels in unexposed workers were quite high.

Using the fluorescence in situ hybridization (FISH) technique to examine specific chromosomes for effects of longer-term benzene exposure, the investigators did not detect differences between the numbers of chromosomal aberrations in exposed and unexposed workers. In contrast, FISH data in the NCI/China study evaluating the same chromosome (chromosome 7) showed increased numbers of aberrations in exposed workers. However, differences in cell culture conditions, probes evaluated, and scoring criteria make it difficult to compare the FISH results between the 2 studies. In addition, the overall frequencies of numerical aberrations (hyperdiploidy) reported in the unexposed control subjects participating in the NCI/China study were unusually high, which complicates comparisons. Although the median exposures of workers in the NCI/China and HEI studies were similar, workers in the NCI study with above-median exposures were exposed to much higher benzene levels than those participating in the HEI study. These higher exposures may have also contributed to the differences between the FISH results of the 2 studies. Using conventional cytogenetic techniques to evaluate all chromosomes, Qu and colleagues found some increases in aberrations in exposed workers compared with controls. These increases were difficult to interpret because they were not linear with recent changes in benzene exposure. However, a more consistent exposure-response relationship was seen when the aberration frequencies were categorized by cumulative benzene exposures.

The investigators evaluated exposure-response effects in the phase 2 subjects combined with the subjects who had been evaluated in phase 1, which was conducted in the previous year. Combined analysis of phase 1 and phase 2 results may have introduced unmeasured confounding because exposures in the 2 phases were measured in different years and at different sites. Further, they used different subjects with much lower exposure levels—by design—in phase 2 than phase 1. Although Qu and colleagues amply addressed many aspects of this issue in the report, the validity of combining data from phases 1 and 2 of the study remains uncertain.

In conclusion, Qu and colleagues' study has validated several biomarkers. Urinary levels of *S*-PMA appear to be the most useful measure of exposure to benzene (detecting changes within a few hours). Blood adducts of benzene and albumin may be useful biomarkers of exposure within days to weeks, but background levels of these adducts are quite high in people not exposed to benzene. Finally, the investigators found that changes in neutrophil levels may be a sensitive and early marker of benzene's toxicity, but further research is needed to confirm this last finding.

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HEI STATEMENT

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

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.

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INVESTIGATORS' REPORT

Validation and Evaluation of Biomarkers in Workers Exposed to Benzene in China

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ABSTRACT

This study was conducted to validate biomarkers for early detection of benzene exposure and effect in 2 phases. The main purpose of phase 1 was to determine whether these biomarkers could reliably detect differences between workers with high exposure levels and unexposed subjects, which is the minimal screening criterion for a biomarker assay. Phase 2 of the study mainly focused on evaluating the exposure-response relation, confounding factors, and sensitivities of biomarkers for low benzene exposures.

The Chinese occupational population studied had a broad range of benzene exposures. On the day of biological sample collection, exposures ranged from 0.06 to 122 ppm with a median exposure of 3.2 ppm. The median of the 4-week mean benzene exposures was 3.8 ppm, and the median lifetime cumulative exposure was 51.1 ppm-years. Compared with benzene levels in collected samples, toluene levels were relatively high, with a median of 12.6 ppm (mean, 26.3 ppm), but xylene levels were low, with a median of 0.30 ppm (mean, 0.40 ppm).

The biomarkers evaluated were urinary metabolites *S*-phenylmercapturic acid (*S*-PMA*), *trans,trans*-muconic acid (*t,t*-MA), hydroquinone (HQ), catechol (CAT), and phenol; albumin adducts of benzene oxide and 1,4-benzoquinone (BO–Alb and 1,4-BQ–Alb, respectively) in blood; blood cell counts; and chromosomal aberrations. Blood cell counts in this population, including red blood cells (RBCs), white blood cells (WBCs), and neutrophils, decreased significantly with increased exposures but remained in normal ranges. Chromosomal aberration data showed significant increases of chromatid breaks and total chromosomal aberrations in exposed subjects compared with unexposed subjects.

Among the urinary metabolites, the levels of *S*-PMA and *t,t*-MA were significantly elevated after benzene exposures. Both markers showed significant exposure-response trends even over the exposure range from 0 to 1 ppm. However, HQ, CAT, and phenol showed significant increases only for benzene exposure levels above 5 ppm. Multiple regression analyses of these urinary metabolites on benzene exposure indicated that toluene exposure, smoking status, and cotinine levels had no significant effects on urinary metabolite levels. A time-course study estimated the half-lives of *S*-PMA, *t,t*-MA, HQ, CAT, and phenol to be 12.8, 13.7, 12.7, 15.0, and 16.3 hours, respectively. Both BO–Alb and 1,4-BQ–Alb showed strong exposure-response associations with benzene. Regression analyses showed that after adjustment for potential confounding by smoking, there was still a strong association between benzene exposure and these markers. Furthermore, the analyses for correlations among biomarkers revealed that the urinary metabolites correlated substantially with each other. The albumin adducts also correlated well with the urinary biomarkers, especially with *S*-PMA. BO–Alb and 1,4-BQ adducts also correlated well with each other ($r = 0.74$).

For benzene exposure monitoring, both *S*-PMA and *t,t*-MA were judged to be good and sensitive markers, which detected benzene exposures at around 0.1 ppm and 1 ppm, respectively. But *S*-PMA was clearly superior to *t,t*-MA as a biomarker for low levels of benzene exposure.

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

Appendices A and B are available at *www.healtheffects.org* or on request from HEI.

This Investigators' Report is one part of Health Effects Institute Research Report 115, 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 Qingshan Qu, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Rd, Tuxedo NY 10987.

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INTRODUCTION

Benzene is an important component of gasoline, is a constituent of engine emissions and combustion products, and is widely used in industry. At high exposure levels, benzene causes progressive degeneration of the bone marrow, aplastic anemia, and leukemia (International Agency for Research on Cancer [IARC] 1982; World Health Organization 1993). Although the effects of low-level exposures are not well understood, there is increasing public concern about environmental exposure to ambient levels of benzene. Benzene is among the components of mobile source emissions identified in the Clean Air Act Amendments of 1990 as toxic air pollutants whose levels need to be reduced.

At present, human health effects resulting from low-level ambient exposures to benzene are generally estimated through extrapolations either from high concentrations to low concentrations or from animal data to humans. The validity of such extrapolations is uncertain, however, and should be addressed in studies of human populations exposed to currently encountered ambient levels of benzene. The difficulties in assessing risks from low-level exposures relate to the lack of sensitive and specific indicators that can be used to correlate personal benzene exposures and biological effects. During the past decades great efforts have been made to develop sensitive biomarkers in animals as well as in humans exposed to relatively high levels of benzene (Inoue et al 1989a; Ducos et al 1992; Bechtold and Henderson 1993; van Sittert et al 1993; Ong et al 1995; Pekari 1995). The biomarkers available so far can serve as measures of total body uptake, biologically effective dose, early markers of biological effects, or markers of susceptibility. However, the applications and predictive abilities of most of these biomarkers have not been fully evaluated at low exposure levels in humans.

Considering the available resources, only the following biomarkers were selected for validation.

BENZENE METABOLITES IN URINE

When humans are exposed to benzene through inhalation, about 12% of the absorbed benzene is exhaled from the lungs and only about 0.1% is excreted unchanged in the urine (Baselt 1980). The remainder is metabolized in the body and excreted primarily through the urine. Therefore, several urinary metabolites of benzene have long been recognized and studied as useful biomarkers of benzene exposure in industrial settings (Inoue et al 1989a; Bechtold et al 1991; Ducos et al 1992; van Sittert et al 1993; Melikian et al 1994, 1999a,b; Boogaard and van Sittert 1995; Ghittori et al 1995, 1999; Ong et al 1995, 1996; Yu and Weisel 1996; Hotz et al 1997; Ruppert et al 1997). Their application in environmental settings is questionable, however, because the levels of benzene exposure in ambient air typically fall within a low range of 2 to 6 ppb (Wallace 1996).

In 1993 the Health Effects Institute funded several studies to support development of more sensitive methods for measuring urinary metabolites, including *t,t*-MA, *S*-PMA, benzene triol (BT), HQ, CAT, and phenol. The goal was to determine whether these metabolites could be used as exposure markers in environmental risk assessment of benzene. Before these endpoints could be accepted as biomarkers in human populations, their use and predictive abilities needed to be validated.

S-PMA and *t,t*-MA

It has been estimated that less than 2% of absorbed benzene is eliminated as *t,t*-MA (Ong et al 1995). It is well established that *t,t*-MA forms in the body primarily through the metabolism of benzene; thus it is fairly specific to benzene exposure (Johnson and Lucier 1992). In addition, *t,t*-MA is produced by biotransformation of *t,t*-muconaldehyde, which is considered to be one of the metabolites responsible for benzene's toxicity (Yardley-Jones et al 1991; Witz et al 1996). Studies have demonstrated a good correlation between urinary *t,t*-MA levels and atmospheric benzene concentrations (Inoue et al 1989a; Bechtold et al 1991, 1993; Ducos et al 1992; Boogaard and van Sittert 1995; Ghittori et al 1995, 1996; Ikeda and Inoue 1995; Ong et al 1995, 1996; Dor et al 1999; Pezzagno et al 1999). In addition, its high chemical stability makes *t,t*-MA reliable and convenient for assessing benzene exposures down to a low ppm level on an individual scale (Ducos et al 1992).

S-PMA is a marker for a detoxification pathway of benzene metabolism and has been found to be useful for monitoring subjects exposed to benzene at low levels (Stommel et al 1989; van Sittert et al 1993; Popp et al 1994). One study indicated that *S*-PMA was superior to *t,t*-MA as a biomarker for low-level benzene exposure: It was found to be more specific, enabling reliable determination of benzene exposure down to 0.3 ppm, and its longer half-life made it more suitable for biological monitoring of operators working in shifts longer than 8 hours (Boogaard and van Sittert 1996). Also, *S*-PMA can be measured simultaneously with *t,t*-MA and thus offers the opportunity to compare it with BO–Alb and 1,4-BQ–Alb adducts in blood without increasing the cost of the study. Therefore, *S*-PMA was included in this study for further validation.

During the past decade, several high-pressure liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC-MS) methods have been developed for measurement of *t,t*-MA and *S*-PMA (Gad-El Karim et al 1985; Ducos et al 1992; Boogaard and van Sittert 1995; Ruppert et al 1995). Generally, HPLC chromatograms of urinary extracts, especially from smokers, contain several compounds that interfere with the exact measurements because they elute in the vicinity of analytes. The GC-MS method eliminates such problems; however, the need to derivatize each analyte makes the GC-MS method less attractive for large-scale epidemiologic studies. Simultaneous quantification of *t,t*-MA and *S*-PMA by liquid chromatography and tandem mass spectrometry (LC-MS/MS) in the current study enhanced both the sensitivity and the specificity of analytes.

HQ, CAT, and BT

In a limited number of studies, HQ and CAT levels showed significant correlations with relatively high benzene exposures (Inoue et al 1989b; Lee et al 1993; Ikeda and Inoue 1995; Ong et al 1995, 1996). Although they appear promising for evaluating occupational exposure to benzene, little information can be found about their use as biomarkers in humans exposed to benzene at low ambient levels. Only one of these studies included measurement of BT (Inoue et al 1989b). HEI investigators have developed a totally new and much more sensitive method to simultaneously measure HQ, CAT, and BT (Melikian et al 1999a), but there are no human data regarding the applicability of this new technique. Therefore, simultaneous measurements obtained by this technique were included for validation in this study.

Phenol

Since it was reported that the major metabolites found in urine after exposure to benzene were phenol and its conjugates (Yardley-Jones et al 1991), phenol has been used as a biomarker for occupational benzene exposure. Its application in populations with low levels of benzene exposure has been questioned, however, because a substantial background level of phenol from sources other than benzene exposure has been found in unexposed subjects (Ong and Lee 1994). Even though phenol did not appear to be a promising biomarker, we still included it in this validation study because it can be compared with the minor metabolites. In addition, most of the previous studies obtained measurements by HPLC, which is not specific for phenol. The method we used to obtain phenol measurements is based on GC-MS and minimizes the interference by other similar chemicals.

BLOOD ALBUMIN ADDUCTS OF BENZENE METABOLITES

Most benzene biomarkers currently available were developed in industrial settings and provide information on exposures that occurred within the previous 8 hours. In assessing occupational and environmental exposures, it is often desirable to use biomarkers which integrate exposures that occur over time periods of at least weeks. Blood protein adducts with benzene-reactive metabolites are among the few markers that fulfill this criterion (Bechtold et al 1992a,b; Bechtold and Henderson 1993; Maestri 1995; Yeowell-O'Connell et al 1998; Hanway et al 2000). Benzene is primarily metabolized in the liver, where it is oxidized by cytochrome P450 (CYP450)–dependent monooxygenases to BO that can bind to macromolecules (Vogel and Gunther 1967). Other benzene metabolites are derived from BO, notably the phenolic metabolites, including phenol (from rearrangement of BO), CAT (by epoxide-hydrolase mediated hydrolysis of BO and subsequent dehydrogenation), and HQ (from a CYP450-dependent oxidation of phenol). CAT and HQ can be oxidized to 1,2-BQ and 1,4-BQ, respectively, which can also bind to macromolecules.

Adducts of toxic chemicals or their metabolites with the blood proteins, hemoglobin and serum albumin, were first proposed as biomarkers of environmental exposures by Ehrenberg and coworkers in the 1970s and now are widely accepted (Ehrenberg et al 1974; Osterman-Golkar et al 1976). After *S*-(2,5-dihydroxyphenyl)cysteine and *S*-phenylcysteine were characterized as major hemoglobin adducts in rodents treated with ^{14}C-benzene (Melikian et al 1992; Bechtold et al 1992a), Bechtold et al first reported elevated levels of cysteinyl adducts attributable to BO (measured as *S*-phenylcysteine) in blood proteins of humans exposed to benzene (1992b). In that study they reported an exposure-response relation between benzene exposure levels and *S*-phenylcysteine derived from albumin among female workers exposed to benzene concentrations of 4.4 to 23 ppm (mean, 13.2 ppm).

After the reports of Bechtold and colleagues (1992b) and Melikian and associates (1992), Rappaport and his coworkers modified the approach and developed more sensitive methods to measure cysteinyl adducts of BO as well as 1,2-BQ and 1,4-BQ with hemoglobin and albumin. Using these assays, they reported exposure-adduct relations in workers exposed to high levels of benzene (1.6 to 329 ppm; median, 31 ppm) (Yeowell-O'Connell et al 1998). Interestingly, unexposed subjects also had high levels of BO–protein and BQ–protein adducts, suggesting important endogenous sources of adducts arising from BO and the BQs.

Given the high levels of exposure to benzene in this study and the presence of background adducts among control subjects, further research is needed to evaluate exposure-adduct relations (BO–Alb and 1,4-BQ–Alb) among persons exposed to lower levels of benzene. It would also be interesting to compare levels of BO–Alb and 1,4-BQ–Alb in blood with the short-lived marker *S*-PMA in urine (van Sittert et al 1993) because both types of biomarkers are presumed to be formed by the binding of BO to cysteine groups. In combination, they may yield more meaningful information about current and cumulative exposures.

CYTOGENETIC BIOMARKERS OF BENZENE EXPOSURE IN BLOOD CELLS

The long latency between exposure and the onset of cancer, combined with other difficulties associated with traditional epidemiologic approaches in identifying human carcinogens, has provided an impetus to develop biological markers for early detection of exposure and effect (IARC 1997). Chromosomal alterations occurring in peripheral blood lymphocytes have been widely used as early-effect biomarkers for monitoring human exposure to carcinogenic agents (Carrano and Natarajan 1988; Tucker et al 1997). Increased frequencies of cytogenetic alterations indicate that an exposure has occurred that is biologically significant and mechanistically related to cancer development (Sorsa et al 1992). Consistent with this, studies have shown that individuals with elevated frequencies of chromosomal aberrations in their peripheral blood lymphocytes are at increased risk of developing cancers, including leukemia (Hagmar et al 1994, 1998; Bonassi et al 1995).

Chronic occupational exposure to benzene has been consistently associated with elevated frequencies of structural chromosomal aberrations in lymphocytes (Sarto et al 1984; Aksoy 1988a). In addition, increased frequencies of numerical aberrations have occasionally been found in these workers (Eastmond 1993; Aksoy 1988a). However, most of these studies observed structural aberrations in workers with current benzene exposure. In contrast, the conventional cytogenetic studies that detected substantial increases in aneuploidy were generally performed on individuals who had exhibited previous bone marrow toxicity, and the studies were initiated some time after exposures had ceased (Pollini et al 1969; Forni et al 1971; Liniecki et al 1971; Pollini and Biscaldi 1976; Ding et al 1983).

Until recently, structural and numerical aberrations in cells were detected by manual scoring of metaphase cells. Conventional cytogenetic studies, although considered the reliable standard, are labor-intensive, require highly skilled personnel, and are prone to other technical problems such as chromosomal loss or poor chromosomal spreading during metaphase preparation. In these studies, cytogenetic information is typically obtained from only a modest number of cells (50 to 100) per individual. Furthermore, these techniques are limited to cells that can divide, such as lymphocytes, and cannot be performed on terminally differentiated cells such as polymorphonuclear leukocytes, which are the end stage in the pathway of the precursor cell primarily affected in benzene-induced leukemia (Aksoy 1988a). These characteristics make conventional chromosomal analysis difficult to use in routine biomonitoring of occupationally exposed workers and very difficult to use for the detection of numerical chromosomal alterations in exposed individuals.

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique that is being increasingly used for detection and quantification of both structural and numerical aberrations in metaphase and interphase human cells. There are many different applications for FISH in the detection of genetic alterations. For human biomonitoring, the most widely applied approach is to use chromosome-specific repetitive DNA sequences as probes to detect changes in chromosome number in cells from exposed persons (for review, see Eastmond and Rupa 1995). In situ hybridization with these probes results in staining of a compact chromosomal region that can easily be detected on metaphase chromosomes or within interphase nuclei. Aneuploidy is determined by counting the number of hybridization regions representing the chromosome of interest within the cell. For a variety of technical reasons, this assay is much more effective at detecting increases in chromosome number (hyperdiploidy and polyploidy) than it is at detecting chromosome loss (for a more detailed discussion, see Eastmond and Pinkel 1990 and Eastmond et al 1995). Note that throughout the current article the term *hyperdiploidy* is used to refer to nuclei containing 3 or more hybridization regions and may also refer to polyploid cells as well as aneuploid cells containing additional chromosomes. Because we evaluated only a single chromosome at a time, these 2 related types of numerical aberration could not be distinguished in the current study. This FISH approach has been successfully applied to identify basal and elevated levels of numerical chromosomal alterations in different cell types of human populations (Robbins et al 1995, 1997; Zhang et al 1996; Martin et al 1997; Ramirez et al 1997; Surralles et al 1997a).

Building on these earlier single-probe FISH studies, Eastmond and associates developed a multicolor FISH strategy to more accurately identify hyperdiploidy in interphase cells and distinguish these cells from nuclei containing breaks affecting the labeled regions (Eastmond et al 1994, 1995; Eastmond and Rupa 1995; Rupa et al 1995). This approach uses a classical-satellite probe that

hybridizes to the large subcentromeric heterochromatin region of chromosome 1 combined with a second α-satellite probe, labeled with a different fluorochrome that hybridizes to an adjacent centromeric region. By evaluating the number and location of colored hybridization regions in the interphase nucleus, hyperdiploidy for chromosome 1 can be distinguished from breakage within the heterochromatic region or between the 2 labeled regions. Additional tandem probe combinations have been developed to allow alterations affecting human chromosomes 9 (9cen-9q12) and 16 (16cen-16q11.1) to also 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 detect hyperdiploidy and breakage affecting these heterochromatic regions in the treated cells or in exposed individuals (Rupa et al 1995, 1997; Conforti-Froes et al 1997; Rupa and Eastmond 1997; Surralles et al 1997b; Schuler et al 1998). Although the term *breakage* is used throughout this report, approximately 10% to 20% of these alterations may represent translocations, inversions, and other types of potentially stable chromosome exchange (Rupa et al 1995). However, their application, sensitivity, and specificity in humans exposed to benzene need to be fully evaluated before they can be used in risk assessment of environmental exposure to benzene.

MATERIALS AND METHODS

OVERVIEW OF STUDY DESIGN

In order to evaluate the capability of candidate biomarkers to index benzene exposure and biological effects, a study design that incorporates several substudies was used. Human data for some biomarkers to be validated were limited. In addition, published studies provided limited data on the ability to detect benzene exposure at levels of current interest.

Several questions need to be addressed in the course of a transitional study to determine the validity and efficacy of an exposure biomarker. We tailored the overall study design to these questions as follows:

1. What is the gross sensitivity of the biomarker? Can it reliably detect differences between persons with high exposure levels and unexposed persons? (If it fails, go no further.)
2. Does the biomarker assay have good reproducibility, and how large is the interindividual variability versus intraindividual variability over time (ie, in repeated samples with exposure levels held constant)?
3. What is the sensitivity of the biomarker at intermediate and low exposure levels? Most environmental exposures currently are at relatively low levels, and the biomarker will be useful only if it can detect exposures at levels that are likely to be encountered.
4. How long is the effective half-life of the biomarker? This information is necessary to interpret results for samples taken at different intervals after exposure or for samples reflecting chronic exposure versus acute exposure.

Phase 1

Preliminary Sensitivity Assessment This initial assessment in phase 1 of the study addressed the first question of whether the biomarkers could reliably discriminate between subjects with high exposures to benzene and unexposed subjects. This is the minimal screening criterion for a biomarker assay. For this assessment we obtained urine samples from 25 unexposed workers and from 25 highly exposed workers at the beginning of their work shift and at the end of that work shift. We also monitored individual benzene exposure levels during the day. This schedule permitted us to analyze the biomarker values immediately after exposure and to subtract preexposure values as an alternative way of looking at the data. (For recruiting of both male and female and smoking and nonsmoking workers, see Examination of Confounding Factors). There is, however, little value in collecting samples before and after a shift for albumin adducts and chromosomal markers since they are more likely to be associated with longer-term exposures. Therefore, we only collected one blood sample from each subject at the end of a shift (this also applies to other similar analyses in phase 1 and 2 of the study).

In all aspects of the study, the samples were handled and assays were performed in a blinded fashion whenever possible, to eliminate investigators' biases. Both exposure samples and biological samples were assigned a unique code at collection. The key linking the exposure and biomarker codes was transmitted to the epidemiologist, who broke the code only after the relevant measurements or assays had been completed.

During phase 1 of the study, we asked 10 of the 25 exposed workers to donate a larger blood or urine sample than was needed for this particular study. We preserved a duplicate set of aliquots for possible reanalysis in a later phase of the study. In addition, we pooled the remainder of the samples and mixed them together to form a large pooled sample of blood or urine that was frozen for use as reference in later phases of the study. This allowed us to determine whether assay results were consistent in the various runs. This is important in two ways. First, if we

found good consistency between the results of assaying a sample from the pool in the phase 1 run and those in phase 2, it would then be possible to statistically pool the phase 1 results with the phase 2 results to gain statistical power and precision. Second, this would provide an important quality control assessment of the biomarker assays. That is, more than expected variability in the pooled-sample analysis within a particular run would be reason to suspect that something went wrong in that run. Likewise, routinely large variability among runs in the pooled-sample results would suggest that the assay needed further developmental work to obtain more consistent results.

Interindividual and Intraindividual Variability This determination of variability was really an extension of the sensitivity assessment previously described. We sought to evaluate interindividual variance in comparison with intraindividual variance in order to determine whether the biomarkers being assessed were fairly constant within subjects over time (given a constant exposure level), which would mean that most of the variability reflected differences in exposure levels. To perform this analysis, we needed multiple measurements on a group of subjects. (According to Fleiss [1986], 15 to 20 subjects are sufficient for continuous-variable data such as we would be analyzing.) We obtained the measurements at consistent times for each subject. We originally planned to collect the samples from 15 subjects (only 11 subjects were persuaded to participate) on 3 consecutive Mondays, one sample at the beginning of the work shift and one at the end, and to monitor the workers' benzene exposure during the day. Analysis of these data was somewhat complex. Again, the basic analytic strategy was to calculate intraclass correlation coefficients between subjects relative to those within subjects over time. Because it was necessary to take into account the workers' exposure levels, which can vary somewhat from day to day, these had to be based on analysis of covariance, treating the daily exposure levels as covariates.

Elimination Half-Lives of Biomarkers (Time Course Study) Since little information is available on the effective half-life of these biomarkers in human subjects, we built a study into phase 1 using a small group of 11 subjects with intermediate to high levels of exposure. We obtained samples before and after the work shift on Friday, on the following Saturday and Sunday mornings (in the factory, but when the subjects were not working), and again before the next work shift began on Monday morning. This permitted us to plot the temporal course of biomarker decay. For biomarkers with relatively fast clearance (eg, those in urine), this should provide a good characterization of the half-life (or of dual short-phase and long-phase clearance kinetics, if present).

For the assays of albumin adducts, however, the procedures outlined might only begin to characterize the temporal pattern because of the long half-lives of the blood proteins (eg, about 20 days for albumin). In such cases we asked these 11 exposed workers to continue their blood donations for up to 2 weeks without further exposures (eg, 2 weeks' vacation). We failed to convince either workers or plant managers to allow us to collect blood samples over such long periods.

Because aneuploidy and chromosomal breakage are markers of biological effects associated with long-term benzene exposure, they were not measured in this section of the study.

Phase 2

Examination of Exposure-Response Relation The exposure-response curve and the biomarker assay's sensitivity (ie, whether it could discriminate between no exposure and intermediate or low levels of exposure) were of major interest for this study. In order to maintain efficiency in terms of the number of measurements required while increasing the precision of results at lower exposures, we weighted the sample toward the lower exposure levels. In particular, we aimed for the following numbers of participants grouped by benzene exposure level: > 15 ppm, 15 subjects (total of 40 subjects including 25 from phase 1 of the study); > 5 to 15 ppm, 25 subjects; > 1 to 5 ppm, 30 subjects; < 1 ppm, 40 subjects; unexposed, 25 subjects (plus 25 more from phase 1).

If the variability in the repeated assays of aliquots from the pooled samples used for reference was suitably small, then the phase 1 data for exposed and unexposed subjects would be incorporated into this data analysis as part of their respective exposure groups.

The regression or correlation analysis of the proposed 135 exposed and unexposed subjects set rather narrow confidence limits on the correlation coefficients generated. The confidence limits on the regression coefficient, given as a percentage of that coefficient, are scaled proportionally according to the ratio of standard deviations of the independent and dependent variables. We performed the calculations assuming that the standard deviations of the 2 sets of variables were identical as a convenient point of reference. Calculations for some sample bivariate correlation coefficients (r) showed the following 95% confidence intervals (CIs): $r = 0.9$, CI = 0.86 to 0.93; $r = 0.8$, CI = 0.73 to 0.85; $r = 0.7$, CI = 0.60 to 0.78. Put in other terms, the 95% CI on the regression coefficient will range from ± 4% of the

coefficient value when $r = 0.9$, to ± 13% of the coefficient when $r = 0.7$. This difference represents excellent precision for the overall exposure-response curve.

Also of interest are the statistical power and precision for comparing the exposure subgroups to the unexposed control group. For example, in comparing 30 subjects in the > 1 to 5 ppm exposure range with the unexposed group, it should be possible to detect a mean difference of 77% of a standard deviation, while for a group with less than 1 ppm exposure, a difference in means of 73% of a standard deviation will be detectable. All sample size or statistical power calculations were performed with $\alpha = 0.05$, 2-sided, and $\beta = 0.2$ (ie, 80% power). If there are unequal variances even after log transformation, then these results should be viewed as only a first approximation. Given that the standard deviation in the unexposed control group is expected to be relatively small (see Evaluation of Biomarker Specificity for Benzene Exposure), this should provide adequate statistical power.

This data set also provided an opportunity to evaluate whether the exposure-biomarker (ie, dose-response) association was essentially linear (or log linear if a log transformation has to be used), or whether there was a nonlinear component to the curve. This was evaluated by adding an exposure-squared component to the basic linear regression model to determine whether the curvilinear term was significant.

Examination of Confounding Factors To minimize any possible effects of age on benzene metabolism and biomarkers, the exposure groups in both phases 1 and 2 were frequency matched according to age. Analyses were conducted to evaluate whether there were differences in either slope or intercept of the biomarker endpoints versus exposure with respect to age.

Although previous studies have not shown differences in the metabolism of benzene according to sex, at least as assessed for certain biomarkers (Ong et al 1995), we included sex as a factor in the design and obtained approximately equal numbers of men and women in most exposure groups. Sex was examined as a covariate in the regression analyses to determine whether there was any main effect or interaction (ie, difference in intercept or slope) according to sex. If there was any suggestion of an effect for either age or sex, that variable was retained as a covariate in all data analyses.

Smoking, one source of benzene exposure, was common among the men in our study population (Dosemeci et al 1994); therefore, we oversampled nonsmoking male subjects at all exposure levels so as to attain an approximate balance between smokers and nonsmokers. The regression analyses also included the presence and intensity of smoking as a covariate in order to control for it and to evaluate whether it was a significant factor. The possibility of an interaction between smoking and benzene exposure level was also examined. Because the self-reporting of smoking status is subjective and has limited validity, we determined urinary levels of cotinine, one of the major nicotine metabolites from cigarette smoking, for evaluating the effects on the biomarkers of both active and passive smoking.

Evaluation of Biomarker Specificity for Benzene Exposure The specificity of candidate biomarkers was assessed with respect to toluene and xylene exposures because of their possible cooccurrence with benzene exposure and potential competitive inhibition on benzene metabolism. Therefore, both toluene and xylene levels were simultaneously analyzed with benzene in the personal exposure samples so that they could be incorporated as covariates when analyzing the association between benzene exposure and the biomarkers to be validated. Another way to assess the specificity of biomarkers is to examine their levels in unexposed subjects. Elevated levels among a fraction of them (ie, a fairly large coefficient of variation [CV]) would indicate lack of specificity in that some unknown exposure or physiologic process is producing the biomarker in question, whereas homogeneously low or null levels of the biomarker would be consistent with specificity.

CHARACTERIZATION OF EXPOSURE IN PARTICIPATING FACTORIES

We recruited subjects from factories in the Tianjin region of China. Phase 1 subjects with benzene exposures worked in a glue factory or a small shoe factory. In phase 2, more subjects with benzene exposures were recruited from a large shoe factory and a sporting goods company. Control subjects without benzene exposure were recruited from a nearby food processing factory (soybean products) for phase 1 and from a flour factory for phase 2.

The glue factory, built in 1972, was an army-owned enterprise. All glue manufacturing and packaging activities were conducted in a building approximately 180 by 30 feet in size (Figure 1). The process used natural rubber from

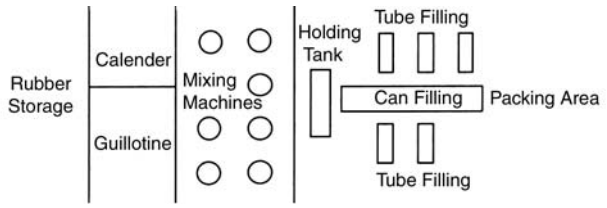

Figure 1. Production lines in glue factory. The glue is made by dissolving natural rubber with an industrial grade of benzene containing approximately 5% toluene.

Malaysia and an industrial grade of benzene containing approximately 5% toluene. To make the glue, the natural rubber was first pressed into a smooth sheet on the calender and cut into small pieces by the guillotine. The small pieces of rubber were put into mixing machines, and then benzene was added from an underground storage tank to dissolve the rubber. The glue was pumped into a holding tank before being distributed to each individual filling machine. There was no mechanical general ventilation. The only local exhaust ventilation was a hood on each filling machine. These hoods were connected to a duct and fan. Though the fan was running, no air motion could be detected. None of the workers used respirators or wore gloves.

The major shoe factory participating in this study was built in 1962 and operated as a state-run enterprise. In 1994 it became a joint venture operated by a company based in Hong Kong. Five workshops located in a 6-story building made heavy protective boot-like shoes for export. Each workshop, with a floor capacity of approximately 120 by 50 feet, had a drying oven through which shoes in the process of assembly moved on a conveyor belt. Workers assigned to gluing tasks removed shoes through doors on the side of the drying oven, performed their task, then put the shoes back on the conveyor belt (Figure 2). Other operations, including sewing, forming, inspection, and packing, were conducted away from the drying oven, along the side of the room. Of the several glues used, only one, a chlorobutyl rubber cement used to glue soles, contained benzene. Some workers used neat benzene to surface treat the shoe soles at the start of the conveyor belt. There was no general ventilation system in any of the workshops. Wall-wide windows on the south and north walls (about 7 feet above the floor) were kept open except during winter. The drying oven in each workshop was covered and connected to a local ventilation system, but there was almost no detectable air motion through the curtain-shielded oven doors. None of the workers used respirators or wore gloves. In addition to this major shoe factory, a small township-owned shoe factory with similar production procedures and exposures was identified during the phase 1 field investigation. A total of 5 subjects were finally recruited from this factory for phase 1 of the study.

The sporting goods company was first operated by the state in 1957 and then reinvested by a Hong Kong company in 1994. Two workshops, the core workshop and the cap workshop, were identified as having operations with benzene exposures. This factory mainly used gasoline-based glue.

The core workshop, located on the third floor of a modern industrial building, was divided into 2 rooms. The outside room (not included in this study) was used to store plain cores, forms that are used as the center material for different types of balls made by the sporting goods company. The cores are made in another workshop without application of any materials containing benzene. The inside room (about 80 by 60 feet, Figure 3) was designated for surface treatment of the cores with gasoline-based glue. Employees working in a chemicals hood manually brushed the cores with glue and then transferred the cores to moving hooks in a sealed dryer with detectable negative pressure. There were no general ventilation systems except one ventilation fan (about 2 feet in diameter) mounted on the wall. None of workers used respirators or wore gloves.

The cap workshop was located on the second floor of the building with a floor capacity of 120 by 60 feet. Working on plain tables, the employees glued small pieces of leather to the dried cores that came from the core workshop. There were 6 tables per row and 9 rows in total in this workshop. There was neither a local exhaust system over the tables nor a general ventilation system in the workshop. Workers used no personal protective measures during their shifts.

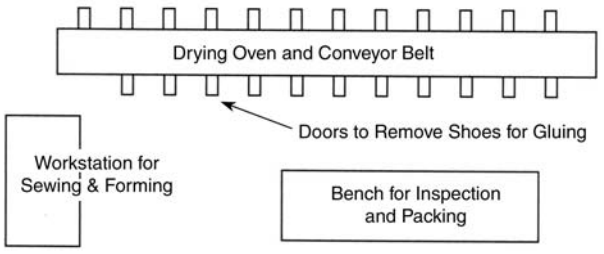

Figure 2. Production lines in shoe factory. One of the glues used, a chlorobutyl rubber cement, contains benzene, and neat benzene is used to treat the shoe soles.

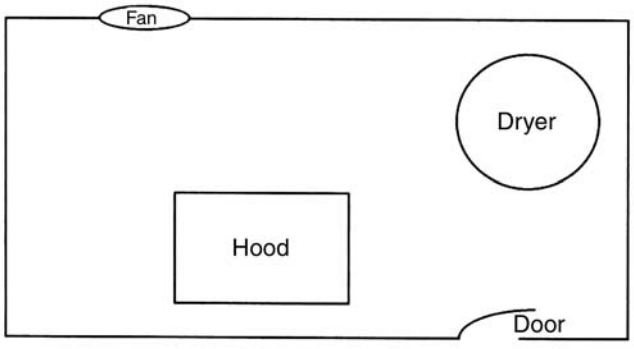

Figure 3. Core workshop in sporting goods company. Workers brush the cores with gasoline-based glue under the hood and then transfer them to hooks in the sealed dryer.

SUBJECT RECRUITMENT AND STRATEGIES FOR SAMPLE COLLECTION

After the factories were identified and their cooperation elicited, the participating subjects were recruited by a 3-step procedure for different study projects (Figure 4). First, our research staff interviewed all exposed workers in the participating factories following a carefully designed questionnaire. The information provided by the workers was then given to the personnel officers of the plants for review and confirmation. Next, workers with at least a 3-year exposure history who had no known diseases and were not currently taking medication were given physical examinations and laboratory tests for liver function and urinary protein levels. Subjects with any diagnosed diseases and those with

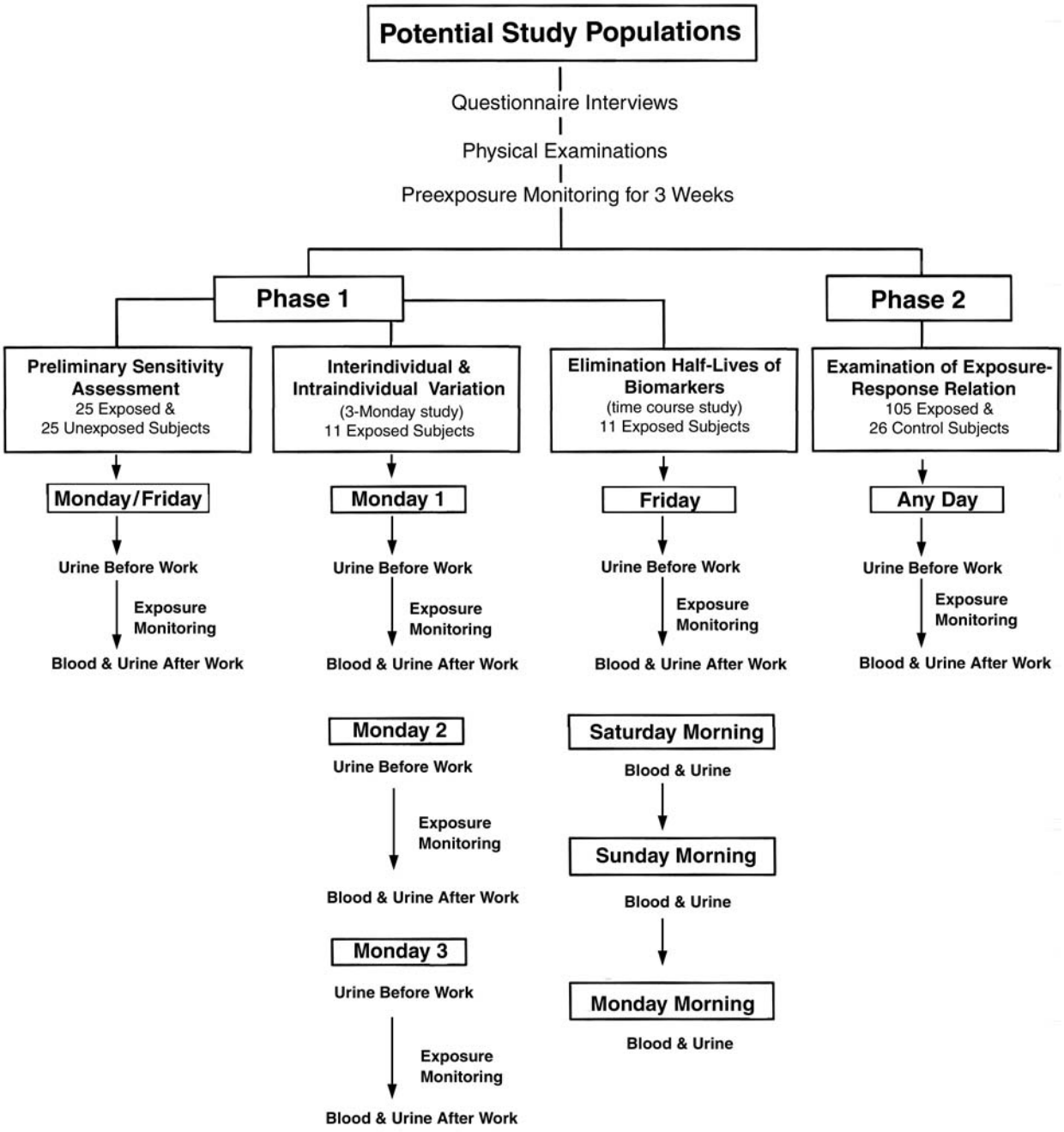

Figure 4. Subject recruitment and sampling strategies. See Table 1 for the factories from which subjects were recruited; all subjects in the 3-Monday and time course studies of phase 1 were from the glue factory.

abnormal liver function or unusually high protein levels in urine were excluded from further consideration. Finally, those workers who passed the physical examination and laboratory tests were monitored with 3M organic vapor monitors to determine their personal benzene exposures at least 3 times within a 1-month period. The mean personal exposure levels of the 3 monitoring sessions were used as criteria for final recruitment into the study. The same exclusion criteria as were used for the personal interview, physical examination, and laboratory tests were also used for recruiting control subjects. All recruited study subjects were informed of the nature of the study as well as its potential benefits and risks. Informed consent was obtained from each subject before study participation.

Table 1 and Figure 4 summarize information on participating subjects and on sampling times and strategies for all studies in phases 1 and 2. On the basis of their individual multiple-exposure levels, 25 exposed workers (20 from the glue factory and 5 from the small shoe factory) were finally recruited into phase 1 of the study to determine whether the biomarkers could reliably detect the difference between highly exposed workers and unexposed subjects. The control subjects (12 men and 13 women) without occupational benzene exposure were enrolled from the nearby food processing factory. Demographic characteristics among exposed and unexposed subjects were similar. The mean age of exposed subjects was 32.1 ± 9.0 years, whereas the mean age of unexposed subjects was 31.7 ± 9.3 years. There were 3 self-reported smokers in the exposed group and 2 in the unexposed group.

To examine the interindividual and intraindividual variation of each biomarker, 11 exposed workers from the glue factory (out of the 25 exposed workers in total) were reassigned for repeated urine, blood, and exposure sampling on 3 consecutive Mondays. The sample collection strategy is shown in Figure 4.

Another 11 exposed workers (4 men and 7 women) were recruited from the glue factory and assigned to a time course study to evaluate the elimination half-lives of biomarkers. Of these workers, 7 were among the 25 participants recruited in the phase 1 study and 4 who were not among the original 25 were recruited separately. All participating subjects were monitored for benzene exposure and asked to provide urine samples before and after work on Friday. Further urine and blood samples were collected on the mornings of the following Saturday, Sunday, and Monday (Figure 4).

To examine the exposure-response relation, another 105 exposed workers (76 from the large shoe factory and 29 from the sporting goods company) and another 26 unexposed subjects from a flour factory were recruited into phase 2 of the study. The demographic characteristics of these exposed and unexposed subjects were similar; the mean age of exposed subjects was 37.2 ± 6.9 years (51.4% were female), whereas the mean age of unexposed subjects was 34.9 ± 4.8 years (53.8% were female). The self-reported smokers accounted for 34.3% of exposed subjects and 30.8% of unexposed subjects. Samples were collected in the same way as for phase 1 (Figure 4).

Table 1. Descriptive Characteristics of All Study Subjects Recruited from Tianjin, China

	Phase 1 (Sampling dates 3/23/1997–4/28/1997)		Phase 2 (Sampling dates 3/12/1998–4/21/1998)	
Characteristic	Exposed	Unexposed	Exposed	Unexposed
Number per factory type[a]	20 Glue 5 Shoe	25 Food processing	76 Shoe 29 Sporting goods	26 Flour
Sex				
Male	11 (44%)	12 (48%)	51 (48.6%)	12 (46.2%)
Female	14 (56%)	13 (52%)	54 (51.4%)	14 (53.8%)
Age (years)[b]	32.1±9.0	31.7±9.3	37.2±6.9	34.9±4.8
Duration of exposure (years)[b]	4.5±2.5	NA	9.7±6.2	NA
Cotinine (μg/g creatinine)[b,c]	238±762	560±1044	714±1277	559±1220
Smoker				
Self-reported	3 (12%)	2 (8%)	36 (34.3%)	8 (30.8%)
Cotinine >100 μg/g creatinine[c]	7 (28%)	8 (32%)	42 (40%)	8 (31%)

[a] Work schedules were 8 hours/day, 5 days/week with no overtime hours except for the glue factory, which was 6–8 hours/day.

[b] Values are expressed as means ± SD. NA = not applicable.

[c] Cotinine levels measured in urine at the time of biological sample collection.

BIOLOGICAL SAMPLE COLLECTION AND HANDLING PROCEDURES

All supplies needed for biological sampling were ordered in the United States and taken to China as check-in luggage by the investigators.

Blood Samples

Blood samples were obtained through venipuncture by Chinese registered nurses in health stations of the participating factories to avoid contamination by the air in the workplaces. First, 5 mL of blood from each subject was collected in a heparinized vacutainer tube with a green cap and labeled with a preassigned 4-digit sample code. Then another 5 mL of blood was collected from the same subject in a yellow-capped vacutainer tube containing citrate and labeled with the same 4-digit code as the green-capped tube. All blood samples were kept at 4°C until they were taken for processing at the local institute (Tianjin Institute of Occupational Medicine for phase 1 or the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine in Beijing for phase 2). The heparinized blood samples were mainly used to make cell preparations in the tissue culture laboratory of the local research institute for later analysis of aneuploidy and chromosomal breakage by the technician at the University of California at Riverside. The citrate-containing samples were centrifuged at 900*g* for 10 minutes to separate plasma from blood cells in the local institute for later albumin adduct analysis. After centrifugation, plasma was carefully transferred into another plain sterile vacutainer tube (labeled with the same number) using a sterile fine-tipped transfer pipette in a tissue culture hood. The separated plasma samples were stored at −20°C to ensure biological stability of the overall samples until they were shipped to the United States. The blood cell component of each sample was also stored at −20°C for DNA isolation in a separate study.

Urine Samples

Before the work shift, urine samples of about 50 mL were collected from each of the recruited subjects into sterile specimen containers. Out of each sample, 14 mL was transferred into a 15-mL cell culture centrifuge tube and labeled with a preassigned code. The transferred urine samples were kept temporarily at 4°C. After collection was complete, all urine samples were taken back to the local institute and stored at −20°C until being shipped to the United States. At the end of the work shift, each worker was asked to provide another urine sample of about 50 mL. These samples were handled in the same manner as the morning samples.

Transportation of Biological Samples

All biological samples except preparations for chromosomal assays were packed with dry ice in sealed boxes and brought back to the United States as check-in luggage by the investigators with permits of exportation issued from the Chinese Ministry of Public Health and permits of importation issued from the US Centers for Disease Control. The cell preparations suspended in Carnoy fixative for chromosomal assays were transported to the University of California at Riverside as check-in luggage by the technician. One of the investigators personally brought the urine samples to the American Health Foundation (Valhalla NY) for analysis. The plasma samples were packed with dry ice and sent by FedEx to the University of North Carolina at Chapel Hill for albumin adduct analysis. A document describing chain of custody was prepared and accompanied all samples during storage and transportation.

PERSONAL EXPOSURE SAMPLING AND RECONSTRUCTION OF HISTORICAL EXPOSURES

The different characteristics of biomarkers in relation to benzene exposures made it necessary to collect 3 categories of exposure data from each participating subject: namely, current-day exposure level, a 4-week mean exposure level (5-week mean for phase 2), and lifetime cumulative exposure level.

Current-Day Exposure

Organic vapor monitors (model 3500; 3M, St Paul MN) were used for sampling of personal benzene exposure throughout the investigation. Current-day exposure sampling was conducted on the days when biological samples were collected. Data from this type of exposure monitoring were mainly used to correlate with urinary metabolites. The field staff used the following stepwise procedures for sampling:

1. Check the expiration date on the packages of monitors before opening the boxes. Discard the monitors if they have expired.
2. Remove the plastic lid from the can and take out the monitor.
3. Before monitoring, record the following information on the plastic can lid and in the notebook: (a) monitor serial number; (b) sampling date; (c) subject identification (ID) number; (d) temperature and relative humidity; (e) compound to be analyzed (benzene, toluene, or xylene).
4. Record starting time on back label of the monitor.
5. Attach 2 monitors (for duplicate sampling) to the center upper chest of each subject.

6. After the sampling period has ended, remove plastic ring and diffusion membrane, snap closure cap firmly onto monitor body, and then inspect the membrane surface for any damage.
7. If there is no damage to the diffusion membrane, record end time and write sample code on back label of the monitor and in the notebook. If the membrane is damaged, repeat sampling.

Four-Week Mean Exposure

Before biological sample collections, all participating subjects were monitored with 3M organic vapor monitors for their personal exposure levels at 1-week intervals for 1 month. All monitored exposure levels, including the current-day level, within a month for each subject (4 samples per subject) were averaged; this averaged value was used to correlate benzene exposure and albumin adduct formation. The sampling procedure was the same as previously described.

Lifetime Cumulative Exposure

In addition to the current-day and 4-week mean benzene exposure data, retrospective exposure data were essential for the validation of aneuploidy and chromosomal breakage. The procedures used in previous studies by one of our collaborators, Dr Guilan Li (Dosemeci et al 1994), were modified in this project for developing retrospective exposure data. In brief, (1) the historical records of job-related benzene exposure (determined by area sampling) for all participating factories were obtained from the local Health and Antiepidemic Stations; (2) the work history of each participating subject, including all job assignments and times and locations in the factory, first were obtained from workers themselves and then were verified by factory personnel staff; (3) historical area exposure records were validated by simultaneous collection of current personal exposure samples (by organic vapor monitors) and current area samples in the factories (by charcoal tubes, the method currently used in China). Because benzene levels obtained by these two different types of sampling agreed with each other, the lifetime cumulative exposure level for each subject could be estimated from individual work history and related historical exposure records of the factory.

QUALITY CONTROL FOR SAMPLE PREPARATIONS IN THE FIELD

Personal Exposure Samples

Preparation of Blanks A blank was a quality control sample monitor used to determine if any background benzene contamination was present owing to the handling of the monitors in the actual work environment. A blank was prepared for each separate set of exposed monitors as follows: (1) at the monitoring site, a monitor was removed from the aluminum can; (2) the diffusion membrane and plastic ring were immediately removed and replaced with a closure cap; (3) a sample code was written on the back label of the monitor as well as in the notebook; (4) the blank was stored and shipped along with the exposed monitors.

Preparation of Spiked Samples Spiked samples were used for quality control purposes as well as for determination of the benzene recovery coefficient, which was used to assess the accuracy and precision of analytic data measured in exposed monitors. The plastic ring and diffusion membrane were removed from a monitor in a clear and uncontaminated laboratory near the factories and then placed on a 2.5-cm-diameter filter paper on a spacer plate. The closure cap was snapped onto the monitor. The amount of benzene to be injected was calculated in milligrams, corresponding to the amount that would be collected by a monitor in the workplace over 8 hours:

$W = K_0 \times C \times t \times (10^{-6}\ \mathrm{m^3/cm^3})$, where

W = amount of benzene injected in milligrams; K_0 = sampling rate of monitor in cubic centimeters per minute; C = mean concentration in milligrams per cubic meter; and t = sampling time in minutes (480 minutes in our study).

The calculated quantity of benzene was injected onto the filter paper through the center port and then the port was recapped. The monitors were allowed to sit for 24 hours in order to allow total transfer of benzene from the filter paper to the sorbent before elution for analysis. The filter paper was removed from the monitor and then the closure cap was again snapped firmly onto the monitor. A sample code was written on the back of the monitor as well as in the log book. The monitor was returned to the original can and shipped along with exposed monitors.

Pooled Biological Samples

To investigate any error inherent in the technical procedures, 10 aliquots of both urine and plasma were made from pooled samples for quality control in both phase 1 and phase 2 of the study. These aliquots were coded to appear like regular study samples, so those performing the analyses were not aware that a given sample was an aliquot from the pooled sample. The procedures for preparing pooled samples are described in the following sections.

Pooling of Blood Plasma Samples

1. Ten subjects were identified from the unexposed control group.
2. In health stations of the participating factories, 5 mL of blood was obtained by venipuncture in vacutainer tubes containing citrate.
3. All blood samples were kept at 4°C until being transported to the local institute.
4. Blood samples were centrifuged at 900*g* for 10 minutes to separate plasma from blood cells in the local institute.
5. Plasma from all 10 tubes was transferred into a sterile 50-mL centrifuge tube with a sterile transfer pipette in a tissue culture hood, and mixed well.
6. Ten aliquots (about 2 mL each) were made from the mixed plasma and put into 10 sterile tubes, each of which was labeled with a different code.
7. These tubes were sealed and stored together with all other plasma samples at −20°C until being shipped to the United States.

Pooling of Urine Samples

1. Urine samples collected at the end of the work shift from subjects exposed to benzene were used for sample pooling preparation.
2. Out of each urine sample of approximately 50 mL, 14 mL was transferred into a 15-mL sterile tissue culture centrifuge tube for analyses of benzene metabolites.
3. Then the leftover urine samples were transferred into a 1000-mL beaker and mixed well.
4. Ten aliquots (about 14 mL each) were made from the mixed urine sample and put into 10 tissue culture tubes, each labeled with a different sample code, as were the regular samples.
5. These pooled samples were sealed and stored together with all other urine samples at −20°C until being shipped to the United States.

ANALYSES FOR BENZENE, TOLUENE, AND XYLENE IN PERSONAL SAMPLERS

To ensure that no personal exposure data would be lost due to unpredictable events during transportation, duplicate samples were collected from each subject; one was analyzed on site at Beijing, China, and the other was brought back to the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine, Nelson Institute of Environmental Medicine at New York University (NYU) for analysis. At both sites, these analyses were conducted by gas chromatography (GC) according to the US National Institute for Occupational Safety and Health standard procedure (Eller 1984). In addition, a quality control procedure was incorporated in the entire analytic process. Briefly, GC calibration was conducted first with 6 working standards. Then prior to beginning sample analysis each day, the GC calibration was confirmed with US National Institute of Standards and Technology (NIST) traceable standards obtained from SUPELCO (Bellefont PA). Three working standards (0.054, 0.544, and 1.087 mg/mL) were injected after every 10th sample, beginning after sample 10 on each day. In addition, every 10th sample, beginning at sample 5 on each day, was repeated (ie, 2 injections were made for precision of analysis). If there was more than 5% variation, the analyses were halted, and the GC was restarted and calibrated again.

BLOOD CELL COUNTS AND DIFFERENTIATION

Blood samples kept at 4°C were delivered for processing within 3 hours after collection to either Tianjin Institute of Occupational Medicine (phase 1) or Institute of Occupational Medicine, Chinese Academy of Preventive Medicine in Beijing (phase 2). The samples were first put at room temperature for 30 minutes and then mixed well for blood cell count and smear preparation. The WBCs, RBCs, and platelets were counted by a cell counter (model PC603, Beijing Analytical Instruments, Beijing, China), which was calibrated daily. In addition, 5 blood smears were prepared from each sample. These were prepared by smearing 5 µL of whole blood onto a glass slide. After air drying, the slides were stored at −20°C under a nitrogen atmosphere until they were shipped to the United States, during which time they were maintained at ambient temperature.

To identify the types of WBCs present, WBC differential analysis and a morphologic evaluation were performed on the blood smears from each study participant. The differential counts were performed by a commercial clinical laboratory (Quest Diagnostics, San Diego CA). Briefly, the slides were stained using an automated slide stainer with Wright-Giemsa stain, and the WBC differential was performed using a ×50 scan of the peripheral smear. A single cytotechnologist manually counted the WBC differential on a total of 900 cells. This comprised 3 sets of differential counts obtained from separate slides: an initial count of 100 cells, and two subsequent counts based on scoring 400 cells per individual.

Calculations of the number of neutrophils, lymphocytes, eosinophils, etc, were based on WBC total counts performed in China and differential counts performed in the United States.

MEASUREMENT OF URINARY METABOLITES

Reagents and Chemicals

[$^{13}C_6$]Phenol ($^{13}C_6$, 99% pure) was purchased from Cambridge Isotope Laboratory (Andover MA). Copper sulfate ($CuSO_4$), copper nitrate [$Cu(NO_3)_2$], urea, sodium nitrite ($NaNO_2$), HQ (> 99% pure), CAT (> 99% pure), BT (99% pure), *o*-nitrophenol and *p*-nitrophenol, 10% palladium-carbon catalyst, and hydrochloric acid (HCl) were bought from Aldrich (Milwaukee WI). Solvents used were HPLC grade from JT Baker (Phillipsburg NJ).

Synthesis of [$^{13}C_6$]*S*-PMA Internal Standard

[$^{13}C_6$]*S*-PMA was prepared from [$^{13}C_6$]aniline by the Gattermann reaction (Rump 1981). In brief, [$^{13}C_6$]aniline (0.5 g, 5.3 mmol) was added to concentrated HCl (0.7 mL), diluted with water (H_2O, 1.5 mL) and HCl (1 mL), and a solution of $NaNO_2$ (1.4 mL, 6 mmol) was added to the resulting suspension while the temperature was kept below 5°C. While stirring, we added the formed phenyldiazonium salt mixture to an *N*-acetyl-L-cysteine solution (7.5 mL, 4.8 mmol), and the resulting orange precipitate was centrifuged. The wet solid of *N*-acetyl-*S*-phenyldiazol-L-cysteine was dissolved in ethanol (6 mL), and after adding freshly prepared copper (0.64 g) and H_2O (14 mL), the suspension was refluxed at 80°C for 1.5 hours. The residue was filtered, washed with hot H_2O, acidified, and extracted with chloroform ($CHCl_3$). After removing the solvent, the crude [$^{13}C_6$]*S*-PMA residue was dissolved in 60% ethanol (2.5 mL); upon adding charcoal (0.25 g), it was boiled and filtered. The [$^{13}C_6$]*S*-PMA was crystallized from aqueous ethanol and characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS). Both the NMR and MS analyses were similar to those reported for *S*-PMA (Melikian et al 1992). The 360-MHz NMR was chloroform-*d* ($CDCl_3$): δ 1.87 (3H, s, CH_3), 3.34 and 3.54 (2H, dd, cys_β and cys_β $J_{\beta\beta}$ = 13.6 Hz), 4.77 to 4.82 (1H, m, cys_α), 6.2 (1H, d, NH), 7.21 to 7.43 (5H, m, aromatic), 12.8 (1H, broad, COOH)]. The electron impact MS spectrum showed ions at *m/z* 245 (M^+) and *m/z* 186 (M-59). The synthesized [$^{13}C_6$]*S*-PMA was more than 98% pure by HPLC, and liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ES-MS/MS) analysis showed no detectable levels of unlabeled *S*-PMA. Unlabeled *S*-PMA was prepared as previously described (Melikian et al 1992).

Biosynthesis of [^{13}C]*t,t*-MA Internal Standard

[$^{13}C_6$]*t,t*-MA as an internal standard has been prepared biosynthetically. Two groups of rats, 3 per group, were given intraperitoneal injections of either 2.6 mmol [$^{13}C_6$]benzene, or 2.6 mmol [^{14}C]benzene (1 mCi/mmol), per kilogram of body weight, in 0.2 mL of corn oil once a day for 3 days. Their urine voids were collected at 0°C during exposure and after exposure on day 4; the urine samples were then stored at −20°C until analysis. [$^{13}C_6$]-Labeled standards were isolated from the urine by the method described previously (Melikian et al 1993). In brief, urine samples were first subjected to solid-phase extraction clean-up procedures, followed by HPLC purification.

Syntheses of [$^{13}C_6$]CAT and [$^{13}C_6$]HQ Internal Standards

[$^{13}C_6$]CAT and [$^{13}C_6$]HQ internal standards were prepared by a method similar to that described for ^{14}C-labeled compounds (Melikian et al 1999a). [$^{13}C_6$]Phenol (0.8 mmol) was dissolved in 1 mL of glacial acetic acid and added to a suspension of $Cu(NO_3)_2 \cdot 3H_2O$; 0.5 mmol in 1 mL of glacial acetic acid at room temperature. After the suspension sat for 1 hour, ice chips were added and the products were extracted 4 times with 5 mL of ether-hexane (3:1). The solvents were removed from the combined extracts under vacuum, and the [$^{13}C_6$]*o*-nitrophenol and [$^{13}C_6$]*p*-nitrophenol products were separated by thin-layer chromatography using hexane-acetone (5:2). The purity of the compounds was determined by HPLC analysis. The yields were 40% for [$^{13}C_6$]*o*-nitrophenol and 28% for [$^{13}C_6$]*p*-nitrophenol.

To prepare [$^{13}C_6$]CAT, [$^{13}C_6$]*o*-nitrophenol (0.2 mmol) was hydrogenated at 40 psi in 10 mL ethanol with 25 mg of 10% palladium-carbon catalyst for 1 hour. The mixture was filtered into 1 mL of 4 N sulfuric acid (H_2SO_4), and most of the ethanol was removed under vacuum. After cooling to between 0°C and 5°C, 1.1 mmol $NaNO_2$ in 1 mL H_2O was added; the mixture was stirred at 0°C for 1 hour. Excess nitrous acid was destroyed by urea; then, 10 mL of a solution of $CuSO_4 \cdot 5H_2O$ (0.05 mol) and cuprous oxide (Cu_2O) (0.3 mmol) was added, and stirring was continued overnight at room temperature. Upon extraction 4 times with 10 mL ether and removal of the solvent, the residue was dissolved in 1 mL methanol (MeOH) and purified by HPLC. The compound eluted at the same retention time as the unlabeled standard; it was collected from the HPLC, dried, dissolved in MeOH, and characterized by its UV spectrum, λ_{max} = 293 nm ($\log\epsilon_{max}$ = 3.4), and by liquid chromatography–mass spectrometry (LC-MS) in the negative ionization mode, which showed a molecular ion at *m/z* 115 $(M\text{-}1)^-$.

For the synthesis of [$^{13}C_6$]HQ, a [$^{13}C_6$]*p*-nitrophenol (0.2 mmol) sample was reduced at 40 psi with H_2 in 10 mL of ether with 15 mg of 10% palladium-carbon for 1.5 hours. The mixture was filtered and the solvent was removed under vacuum. The residue was immediately dissolved in 2 mL of 6 N H_2SO_4, then cooled to 5°C, and 0.6 mmol $NaNO_2$ in 2 mL of H_2O was added. The mixture

was stirred at 5°C for 3 hours. Urea was used to destroy excess nitrous acid, and then 15 mL of a solution of $CuSO_4 \bullet 5H_2O$ (0.07 mol) and Cu_2O (0.25 mmol) was added. Stirring was continued overnight at room temperature. The mixture was extracted 4 times with 10 mL of ether; after removal of the solvent, the residue was dissolved in 1 mL of MeOH and purified by HPLC. The compound eluting at the appropriate retention time was collected from the HPLC, dried, dissolved in MeOH, and characterized by its UV spectrum, λ_{max} = 225 nm ($\log\varepsilon_{max}$ = 3.7) and λ = 293 nm ($\log\epsilon$ = 3.43), and by LC-MS in the negative ionization mode, which showed a molecular ion at *m/z* 115 $(M\text{-}1)^-$.

LC-ES-MS/MS Analysis of *S*-PMA and *t,t*-MA

Simultaneous measurements of *S*-PMA and *t,t*-MA were obtained by LC-ES-MS/MS using a method described previously (Melikian et al 1999a,b). Urine samples (1 mL), spiked with 15 ng of $[^{13}C_6]$*S*-PMA and 30 ng of $[^{13}C_6]$*t,t*-MA as internal standards, were passed through a strong anionic exchange cartridge that was preconditioned with 5 mL of MeOH and 5 mL of H_2O. The cartridge was eluted with 3 mL of H_2O (fraction 1), then with 3 mL of 5 mmol phosphate buffer, pH 7 (fraction 2), and 3 mL of 1% aqueous acetic acid (fraction 3), and finally the analytes of interest were eluted with 4 mL of 10% aqueous acetic acid (fraction 4). Analytes eluted as fraction 4 were extracted 3 times with 5 mL of ethyl acetate; these extracts were combined and evaporated to dryness under N_2 at room temperature. The residue was dissolved in 150 µL of MeOH–1% aqueous acetic acid (20:80 vol/vol), and a 30-µL sample of this solution was analyzed by LC-ES-MS/MS–selected reaction monitoring.

The HPLC features included a Waters model 600 pump, a Rheodyne model 7120 injector, and a Phenomenex Ultramex 5-µ C-18 narrow-bore column (250 × 2.0 mm). A preinjector splitter was utilized to reduce the flow rate from 0.9 mL/min (HPLC pump) to 160 µL/min. A linear gradient from 80% solvent A (0.5% aqueous acetic acid) and 20% solvent B (MeOH) to 100% solvent B over 5 minutes was employed in the elution program for analysis of *S*-PMA and *t,t*-MA.

The HPLC was interfaced with a Finnigan (San Jose CA) TSQ 700 triple-stage quadrupole mass spectrometer via an electrospray source. The mass spectrometer was operated in the negative ion mode. The spray voltage was 4.1 kV; the capillary temperature, −20°C. The liquid flow was introduced into the mass spectrometer at a rate of 160 µL/min without postcolumn splitting. For selected reaction monitoring, the first quadrupole (Q1) was used to select the precursor ion for the reaction and pass it on to Q2, where fragment ions were produced via collision with argon. The product ion of the reaction was monitored by Q3. The argon gas pressure in the collision cell (Q2) was adjusted so that the precursor beam suppression was approximately 75%. A Digital Equipment Corporation Station Number 5000-120 computer was used to control the instrumentation, data acquisition, and data processing.

In the pooled urine samples, the CV was 17% for *S*-PMA at a concentration of 50.7 µg/L urine (n = 9), 12% for *t,t*-MA at a concentration of 2.1 mg/L urine, 12% for creatinine at a concentration of 0.72 g/L urine, and 202.8% for cotinine at a concentration of 1235 µg/L urine.

LC-ES-MS/MS Analysis of HQ, CAT, and BT

The HQ and CAT were quantified and BT was estimated by the LC-ES-MS/MS assay developed previously (Melikian et al 1999a). In brief, we added 50 µL of $[^{13}C_6]$CAT (50 ng/µL) and 45 µL of $[^{13}C_6]$HQ (26 µg/µL) internal standard solutions to 1-mL aliquots of samples. The liquids were vortex-mixed and subjected to acid hydrolysis. After 350 µL of 20% double-distilled HCl was added to the urine samples, they were incubated at 90°C for 1.5 hours. After cooling, 300 mg of sodium sulfate was added, and the mixtures were extracted twice with 2 mL of ether. The solvent was removed under a stream of N_2 at room temperature, and the samples were stored at −20°C until they were analyzed.

Each residue was dissolved in 200 µL of MeOH. Thirty-microliter aliquots of this solution were transferred into automatic injector vials and mixed with 2 volumes of sodium bisulfate solution (2 mg dissolved in 1 mL of H_2O, to prevent autooxidation of the analytes), flushed, and kept under N_2. A 20-µL aliquot was analyzed by LC-ES-MS/MS using the automatic injector. The HPLC features included a Waters model 600 pump, a Rheodyne model 7120 injector, and an Eclipse XDB C-18 cartridge, 3.5-µm (30 × 2.1 mm), from MAC-MOD Analytical (Chadds Ford PA). A preinjector splitter was utilized to reduce the flow rate from 0.9 mL/min (HPLC pump) to 160 µL/min. The HPLC program consisted of isocratic elution with MeOH-H_2O (50:50) for 10 minutes, followed by a linear gradient from 50% MeOH to 100% MeOH over 5 minutes, and kept at 100% MeOH for another 25 minutes. The HPLC was interfaced with a Finnigan TSQ 700 triple-stage quadrupole mass spectrometer. The MS spectra of BT, HQ, and CAT indicated that these analytes readily formed noncovalent dimers under mild electrospray conditions. Relatively high capillary and tube lens values of 45 and 115 V, respectively, had been applied to break the dimers. Because HQ and CAT are chromatographically separated by the HPLC column, the $(M\text{-}1)^-$ ions of HQ and CAT at *m/z* 109 and

ions at *m/z* 115 for [$^{13}C_6$]HQ and [$^{13}C_6$]CAT were selected in the Q1 analyzer; ions produced at *m/z* 108 and *m/z* 114, respectively, were monitored by the second analyzer. Benzene triol decomposes more extensively upon collision activation. The most abundant ion, under typical collision-activated decomposition conditions, is produced by the loss of H_2O and CO, and the transition corresponding to this reaction, from the $(M-1)^-$ ion (125) to *m/z* 79, is used to monitor BT. Levels of BT were estimated using [$^{13}C_6$]HQ as the internal standard.

GC-MS Analysis of Phenol

Sample Preparation After 0.4 mL of concentrated HCl solution was added to 2.0 mL of a thawed urine sample, the mixture was incubated for 1.5 hours at 95°C in a loosely stoppered glass test tube in a temperature-controlled water bath. The sample was allowed to come to room temperature and spiked with 10 µL of a solution containing internal standard (phenol-D5, 40 mg/mL aqueous solution). About 0.5 g of sodium chloride and 1.0 mL of diethyl ether were added to the solution. The mixture was kept in an ice bath for 5 minutes and shaken vigorously for 1 minute. The ether extract was transferred to a clean, cold test tube containing a few milligrams of anhydrous sodium sulfate. The extraction procedure was repeated with a fresh 1-mL volume of ether. The combined, dried ether extracts were transferred into a glass vial with a tight Teflon cap seal, labeled, and stored in a freezer at −20°C until the GC-MS analysis.

GC-MS Analysis A Hewlett-Packard GC unit, model 5890A, with a HP-1MS crosslinked methyl siloxane capillary GC column (30 m × 0.25 mm × 1 µm), was employed and linked to a VG 70SE mass spectrometer for phenol separation and quantification. The column temperature was increased from 50°C to 260°C in 13 minutes after the initial isothermal period of 2 minutes. The GC injector was maintained at 100°C. The mass spectrometer was operated at the unit mass resolution in an electron impact ionization, selected-ion mode and was tuned and calibrated using perfluoro kerosene. Two masses were monitored throughout each GC-MS run corresponding to the molecular ions of phenol and phenol-D5. Ratios of the peak areas were calculated from the chromatograms and used in the data analysis. Quantification was accomplished by fitting these ratios into a calibration curve, constructed on the basis of data obtained from the phenol-spiked urine.

Determination of Creatinine and Cotinine Levels

Urinary creatinine level was determined with a Kodak Ektachem 500 Computer-Directed Analyzer to provide a point of reference for adjusting the concentration of analytes in urine samples according to variations in liquid uptake among subjects. Cotinine, one of the major metabolites of nicotine, was selected as an indicator of smoking status for the study subjects and quantified by radioimmunoassay at the American Health Foundation's Clinical Biochemistry Facility according to previously described methods (Haley et al 1983; Melikian et al 1994).

MEASUREMENT OF ALBUMIN ADDUCTS OF BO AND 1,4-BQ

Processing of Samples

A total of 247 plasma specimens were obtained, each coded with a 4-digit sample code unrelated to the subjects. After all samples had been processed and adduct levels had been reported, subject ID codes were obtained. At that point it was determined that plasma from 1 subject (sample code 6304, ID 121) had not been received by our laboratory and thus it was not included in our analysis. For quality assurance purposes, some plasma specimens were chosen at random for duplicate assays (76 specimens for BO–Alb and 77 for 1,4-BQ–Alb) and some assays were chosen at random for duplicate gas-chromatographic injections (108 assays for BO–Alb and 61 for 1,4-BQ–Alb).

Isolation of Albumin from Plasma

Albumin was isolated from plasma as described in Lindstrom et al (1998). Briefly, an equal volume of saturated ammonium sulfate [$(NH_4)_2SO_4$] was added to plasma to bring the final concentration to 50%. This mixture was centrifuged at 900*g* to remove immunoglobulins. The supernatant was exhaustively dialyzed (Spectra-Pore, 12,000 to 14,000 molecular weight cutoff) 4 times against 3.5 L of deionized H_2O at 4°C, and the dialysate was lyophilized to a constant weight.

Analysis of BO–Alb and 1,4-BQ–Alb Adducts

Cysteinyl adducts of BO and 1,4-BQ with serum albumin were measured according to the assay described by Waidyanatha and colleagues (1998) with slight modifications. The corresponding 1,2-BQ–Alb adduct was also detected in these samples but could not be reliably quantified owing to the lack of an appropriate internal standard. (We have observed that 1,2-BQ–Alb is unstable under the conditions of our assay.)

Briefly, 10 µg of [$^2H_3$]1,4-BQ–Alb (in 10 mM desferoxamine and 100 mM ascorbic acid) and 10 pmol [$^2H_5$]*S*-phenyl cysteine were added to 5 to 10 mg of albumin, and samples were brought to complete dryness in a vacuum oven (70°C to 80°C, 15 mm Hg). The dried protein was reacted with 750 µL of trifluoroacetic anhydride and 20 µL of methanesulfonic

acid at 100°C for 40 minutes, and the unreacted anhydride was removed under a gentle stream of N_2. Then 1 mL of hexane was added to the residue, and the hexane layer was washed once with 1 mL of 0.1 M Tris buffer (pH 7.5) and twice with 1 mL of deionized H_2O. After concentrating the samples to 200 µL, an aliquot of 2 to 3 µL was analyzed by GC-MS in the selected ion-monitoring mode. Standards were prepared in a similar manner to the samples except that *S*-phenylcysteine and *N*-acetylcysteine–bound 1,4-BQ, mixed with 0.5 to 1 mg of albumin, were used instead of the adducted protein.

Samples were analyzed by GC-MS in negative ion chemical ionization mode using a HP 5980 series II plus gas chromatograph coupled to a HP 5989 B MS engine. Helium was used as the carrier gas at a flow rate of 1 mL/min, and methane was used as the chemical ionization reagent gas at 1.5 Torr. The injector and MS transfer-line temperatures were 250°C and 280°C, respectively. For the analysis of 1,4-BQ–Alb, a DB-5 fused silica capillary column (30 m, 0.25-mm internal diameter, 1-µm film thickness; J & W Scientific) was used with an ion source temperature of 150°C. The GC oven temperature was held at 75°C for 3 minutes, then increased at 8°C per minute to 120°C, and held at that temperature for 18 minutes. For the analysis of BO–Alb, a DB-5 fused silica capillary column (60 m, 0.25-mm internal diameter, 0.25-µm-film thickness; J & W Scientific) was used with an ion source temperature of 100°C. The GC oven temperature was held at 50°C for 3 minutes, then increased at 2.4°C per minute to 80°C, followed by 0.9°C per minute to 90°C. In both cases, late-eluting compounds were removed by increasing the oven temperature by 50°C per minute to 250°C, where it was held for 10 minutes.

The reaction of adducted protein with methanesulfonic acid and trifluoroaceticanhydride releases *O,O′,S-tris*-trifluoroacetyl-hydroquinone (HQ-*S*-TFA) from the 1,4-BQ adduct and phenyltrifluorothioacetate (PTTA) from the BO adduct. For the quantitation of adducts, the following ions were monitored in selected ion monitoring mode: for HQ-*S*-TFA, *m/z* 333; for [$^2H_3$]HQ-*S*-TFA, *m/z* 336; for PTTA, *m/z* 206), and for [$^2H_5$]PTTA, *m/z* 211. The ions *m/z* 333 and 336 correspond to loss of a trifluoroacetyl group from triderivatized HQ-*S*-TFA and [$^2H_3$]HQ-*S*-TFA. The quantitation was based on peak areas relative to the isotopically labeled internal standard. The response ratio of HQ-*S*-TFA to [$^2H_3$]HQ-*S*-TFA was assumed to be 1. The response ratio of PTTA to [$^2H_5$]PTTA was determined to be 0.8 from the standard calibration.

FISH AND CONVENTIONAL CHROMOSOMAL ABERRATION ASSAYS

Blood samples obtained from each donor were processed in the following manner: For the G_0 lymphocyte and granulocyte preparations, 1 mL of 0.75 M potassium chloride (KCl) was mixed with 100 µL of blood and incubated for 30 minutes at room temperature. Then 150 µL of a freshly made methanol–acetic acid (3:1) solution was added, and the mixture was centrifuged at 133*g* for 5 minutes using a microcentrifuge. The supernatant was removed, and the pellet was resuspended in methanol–acetic acid (3:1) fixative 3 times. The samples were stored at 4°C until being transported to the United States.

Lymphocyte cultures were established by adding 0.5 mL of whole blood to 5 mL RPMI 1640 cell culture medium. The medium also contained 5% iron-supplemented calf serum, 5% fetal calf serum, a penicillin-streptomycin solution (100 U and 100 µg/mL), phytohemagglutinin (2.36%, M form), and L-glutamine (2 mM). The cultures were incubated in a humidified carbon dioxide (CO_2) incubator with 5% CO_2 at 37°C in the dark until harvest. Prior to harvest at 51 or 72 hours, cells were treated with a 0.075 M KCl hypotonic solution for 30 minutes at room temperature, fixed 3 times in methanol–acetic acid (3:1), and stored at −20°C until being transported to the United States. For the metaphase preparations, colcemid (final concentration, 0.05 µg/mL) was added to the cell cultures at 48 hours—3 hours prior to harvest at 51 hours.

After arrival in the United States, all cell suspensions were refixed with methanol–acetic acid (3:1), one or more times as needed, and dropped onto cleaned glass slides. After air drying, the slides were stored in a freezer-grade Ziploc type of plastic bag under a nitrogen atmosphere at −20°C until use.

Probes, Probe Generation, and Labeling Conditions

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

For generation of an α-satellite probe specific to chromosome 7, we used an α-satellite 20mer primer specific to chromosome 7, designated Asat7c, 5′-AGC GAT TTG AGG

ACA ATT CT-3′, and a human centromere-specific 30mer primer named SO-aAllCen, 5′-GTT TTG AAA CAC TCT TTT TGT AGA ATC TGC-3′. DNA from the somatic cell hybrid GM10791, a hamster cell line containing human chromosome 7 (Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden NJ) was used as a template. The polymerase chain reaction (PCR) conditions employed were similar to those described in Hasegawa et al (1995). After hot starting the reaction by denaturing the DNA at 94°C for 5 minutes and then adding 5 U of *Thermus flavus* (*Tfl*) polymerase (Epicentre Technologies, Madison WI), amplification was performed for 30 cycles of 30 seconds at 94°C, 30 seconds at 42°C, and 1 minute at 72°C, followed by 1 cycle of 15 minutes at 72°C. The PCR amplification products were nick-translated according to the protocol provided with the DNA polymerase/DNase I enzyme mixture (Amersham Life Sciences) (Hasegawa et al 1995). Digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis IN) was used to label the probes by nick-translation.

FISH

All hybridization procedures were performed using previously described procedures (Trask and Pinkel 1990). Briefly, slides were immersed in a solution of 70% formamide and 2× standard saline citrate (SSC) for 5 minutes at 70°C, dehydrated in an ethanol series, and placed on a slide warmer at 37°C. Multicolor FISH with the α-satellite probe and classical-satellite probe for chromosome 1 was performed in 55% formamide, 10% dextran sulfate, 1× SSC, using 1 μg of sheared herring sperm DNA, 1 μL of digoxigenin-labeled α-satellite probe for chromosome 1, 20 to 100 ng of the Cy3-labeled classical-satellite probe for chromosome 1, and deionized H_2O as required, all in a volume of 10 μL. Posthybridization washes were performed in 60% formamide and 2× SSC 3 times for 5 minutes each at 41°C, and 1 time in 0.1× SSC at 41°C. Hybridizations using the chromosome 7 α-satellite probe were conducted using the same hybridization and washing conditions. The digoxigenin-labeled α-satellite probes for chromosomes 1 and 7 were detected using a mouse antidigoxigenin immunoglobulin G (IgG) (Boehringer Mannheim; 3.2 μg/mL in PX buffer [0.1 M phosphate buffer, pH 8.0; 0.5% (wt/vol) Triton-X-100] with 5% nonfat dry milk [PXM]), followed by an amplification round with digoxigenin-conjugated sheep antimouse antibody (Boehringer Mannheim; 20 μg/mL PXM) and a third layer consisting of fluorescein isothiocyanate (FITC)–conjugated sheep antidigoxigenin IgG (Boehringer Mannheim; 20 μg/mL in PXM). To counterstain the DNA for the tandem chromosome 1 assays, 4′,6-diamidino-2-phenylindole (DAPI) (0.1 μg/mL in phenylenediamine antifade) was used. For the single labeled chromosome 7 probe, propidium iodide (0.5 μg/mL in phenylenediamine antifade) was used to counterstain the DNA.

All slides were scored using a Nikon fluorescence microscope at ×1250 magnification. The frequencies of alterations were determined by scoring 1000 cells per individual from coded slides for each cell type analyzed. Cells were identified by nuclear morphology and are reported by the dominant cell type scored. Included among the coded slides were coded positive control slides for breakage (radiation) and hyperdiploidy (diethylstilbestrol-treated) as well as untreated negative control slides. For the G_0 lymphocyte and granulocyte studies, G_0 lymphocyte and granulocyte cell preparations irradiated in whole blood at 0 and 2 Gy were included. For the analyses of lymphocytes cultured for 51 hours with the tandem probes, untreated, irradiated (2 Gy) and diethylstilbestrol-treated (10 μM) cultured lymphocyte slides were used. For the studies of lymphoctyes cultured for 72 hours to detect aneuploidy, slides prepared from untreated and diethylstilbestrol-treated cultures were employed.

Fluorescence filters and scoring criteria for the FISH studies have been described previously (Eastmond et al 1994). A blue filter (Nikon B-2A; excitation at 450 to 490 nm, emission at 520 nm) was used to simultaneously make visible the FITC-labeled chromosome 7 α-satellite probe and the nucleus counterstained with propidium iodide. For the multicolor FISH analyses, a triple-band-pass filter (Chroma Technology Corp, Brattleboro VT; #P/N 61002) was used to simultaneously make visible the yellow-green (FITC; α-satellite), red (Cy3; classical satellite), and blue (DAPI; DNA counterstain) signals. In the case of a cell with more than 2 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 Corp; #31004; excitation at 540 to 580 nm, emission at 600 to 660 nm) for the Cy3 signals. Hybridization regions comprising 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. However, a nucleus comprising 3 hybridization regions in which 2 contained adjacent red and yellow fluorochromes and a third region contained only a Cy3-labeled area was scored as containing 2 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-satellite and classical-satellite

probes was scored as breakage between the hybridization regions targeted by the DNA probes. Finally, hybridization regions appearing as doublets or diffused signals were scored as one hybridization region.

Giemsa Staining and Analysis of Metaphase Chromosomes from 48-Hour Lymphocyte Cultures

Slides were removed from the freezer and held at room temperature for at least 1 day prior to staining. The cells were stained by placing the slides in freshly made Giemsa staining solution made with Gurr buffer (GIBCO-BRL) for 3 to 5 minutes, rinsing 2 times with deionized H_2O, and allowing them to air-dry. One hundred well-spread metaphase cells containing at least 45 chromosomes were scored per individual for structural aberrations using a brightfield microscope with a ×100 lens according to the International System for Human Cytogenetic Nomenclature guidelines. For numerical aberrations, the number of metaphase spreads containing 44 or fewer chromosomes, 45 chromosomes, and 47 or more chromosomes, as well as those exhibiting polyploidy, were recorded for each sample.

STATISTICAL ANALYSIS

Clarifications for Data Being Analyzed

The analyses of benzene and toluene in personal samplers collected from nominally "unexposed" subjects were all below the lowest detection limit (about 0.01 ppm for 8-hour sampling period). Therefore, random values (assuming a uniform distribution over the range 0 to 0.01 ppm) were generated for each subject's levels of exposure to benzene and toluene. These values were used for all exposure-response analyses, other than the cumulative exposure analyses, for which 0 was used.

One individual among those providing benzene exposure data had exposure levels more than twice as high as anyone else. Because this observation had the potential to be highly influential in regression analyses, this influence was lessened by arbitrarily reassigning his exposure values to be 20% greater than the next highest value. In addition, because the exposure data were right-skewed, a semiparametric analysis based on exposure ranks and outcome-variable ranks was conducted to corroborate the parametric analyses.

Because of the potential for underreporting of smoking, we compared the cotinine distributions for self-reported smokers and nonsmokers and found several outliers among the self-reported nonsmokers that produced appreciable overlap with the self-reported smokers. On the basis of the lower limits of the distribution among self-reported smokers and the outliers among the self-reported nonsmokers, we decided to assign all self-reported non-smokers who had cotinine levels above 100 μg/g creatinine as smokers. No self-reported smokers were reclassified.

Comparability of Data for Phase 1 and Phase 2

As planned in our study design, the data collected from the phase 1 preliminary sensitivity assessment conducted in 1997 would be incorporated into the phase 2 data obtained in 1998 for examining the exposure-response relation, if comparable. Before combining the data collected in phases 1 and 2 of the study, it was important to evaluate the comparability of the biomarker measurements over those 2 years. Since by design the exposure levels were much higher in phase 1 of the study than in phase 2, the comparisons were limited to the unexposed control groups in the first set of analyses (Table A.1 in Appendix A gives the results of this comparison). The phase 1 and phase 2 groups were comparable with regard to demographics and lifestyle. They did not differ in age, sex, or current smoking status. Although there were several statistically significant differences between the 2 groups with regard to the urinary biomarkers (*t,t*-MA, *S*-PMA, and phenol), these differences were small compared with the range of values in the exposed groups, so are probably not a source of concern. There were few differences between the 2 years with regard to blood cell counts or chromosomal aberrations. All in all, the differences in the biomarker measures were few and mostly small.

To further evaluate the comparability of the phase 1 and phase 2 data, outcome variables for subjects who had exposure levels in a comparable range for phase 1 and 2 (6 to 20 ppm for 4-week mean benzene exposure for the blood-based variables, or 6 to 30 ppm for current-day exposure for the urinary metabolite variables) were compared. Data from phase 1 and 2 were compared using analysis of covariance to control for exposure levels. These analyses included 9 phase 1 subjects and 23 phase 2 subjects for the variables in blood samples, and 14 and 15, respectively, for the variables in urine samples. For the 31 outcome variables in this comparison (7 blood counts, 2 albumin adduct counts, 13 indications of chromosomal aberration, and 9 urinary metabolite variables [after-work, before-work, and after-work minus before-work variables for *S*-PMA, *t,t*-MA, and phenol]), 7 showed a statistically significant difference. The differences were not consistent, however: phase 1 after-work values were higher than phase 2 values for chromatid gaps, hypodiploidy (45 chromosomes), aneuploidy (45 or 47 chromosomes), and *t,t*-MA, but lower than phase 2 values for eosinophils, enhanced hypodiploidy (< 45 chromosomes), and *S*-PMA. In addition, some of the variables were intrinsically correlated with each other (eg, *S*-PMA levels after work, before

work, and after minus before work), so there were fewer than 7 independent significant results.

There were no clear indications of consistent differences between the phase 1 and phase 2 subjects, so combining the 2 data sets for analysis seemed to be justified. In general, it was not thought appropriate to control for phase (year) of the study in the analysis of the data because the exposure levels were much higher in the first year of the study (phase 1 mean, 35.5 ± 39.5 ppm) than in the second year (phase 2 mean, 5.2 ± 7.3 ppm), so if year were included in the analysis, it could potentially severely confound the exposure level and give distorted results with respect to exposure effects. However, a series of methodologic analyses indicated that its effects were relatively small, so it was not viewed as a serious concern and was not included in most analyses. Therefore, the analysis of exposure-response relations was conducted, and is reported in this report, using the combined data.

Statistical Methods

Essentially, the same statistical methods were used for the combined data for both phase 1 and phase 2 as had been used for the phase 1 data alone. All analyses were conducted using SPSS (1994) or SAS (2000) statistical software. When appropriate, the continuous outcome variables were analyzed using a logarithmic or square-root transformation. For the chromosomal aberration counts, the square-root transform $(x + 3/8)^{1/2}$ was applied. This transformation is recommended to achieve approximate normality and variance stabilization in count data (Zar 1984). For the several chromosomal aberration variables for which there were very few nonzero observations, no analyses are presented. The urinary benzene metabolite variables *S*-PMA, *t,t*-MA, HQ, CAT, and phenol were measured as continuous variables but proved to be right-skewed. A logarithmic transformation ($\ln[x + 1]$) was therefore applied to these variables for analysis. Similarly, a logarithmic transformation was applied to a few skewed differential blood counts (monocytes, eosinophils, and basophils) and to the 2 albumin adduct counts.

One-way analysis of variance (ANOVA), including a contrast to evaluate trends, was applied to compare multiple ordered exposure groups. The trend contrast scores consisted of successive values incremented by unity and centered on 0 (eg, −2, −1, 0, 1, 2). As such, it was a nonparametric trend test but did not take into account the relative differences between the mean exposure levels. Multiple regression was used to analyze benzene exposure as a continuous variable that modeled the actual measured exposures.

The multiple regression analyses routinely incorporated several covariates as potential confounders, including sex, age, cotinine level, and toluene exposure. Because of the right skewness of the cotinine and toluene values, logarithmic transformations of these variables were used in the analyses. In addition, subanalyses were performed to evaluate the impact of potential confounding variables on the results, especially in the case of toluene, which at high levels can compete with benzene for binding sites.

For urinary metabolite measures, the primary benzene exposure variable was the personal exposure measurement on the day of the urine sample collection (current-day benzene exposure), while the main outcome variables were the urinary metabolite levels at the end of the workday and the difference scores (the after-work measurement minus the before-work measurement).

Benzene exposure variables used for the primary analyses of blood cell counts, albumin adducts, and chromosomal aberrations were the mean of several personal measurements over a 4-week period (hereafter called "4-week mean exposure"). These included the measurement on the day of biological sample collection plus measurements in the preceding 3 to 4 weeks. Of the 130 benzene-exposed workers, 117 (90%) had 4 measurements, 11 (8.5%) had 3 measurements, and 2 (1.5%) had 2 measurements. The blood cell count and chromosomal aberration data were also analyzed in relation to estimated lifetime cumulative occupational exposure to benzene (ppm-years).

For analysis of albumin adducts, statistical procedures employed SAS software (SAS 2000). Because of obvious skewness and heteroscedascity, analyses were conducted following natural logarithmic transformations of measurements of exposure and adduct levels. Subject-specific means of the logged measurements were used to estimate individual benzene exposures for the 130 exposed workers (2 to 6 personal measurements per subject). Unexposed control subjects were assigned an exposure of 0 ppm benzene for regression analyses. Logged adduct measurements were likewise averaged by subject according to a nested model in which GC-MS injections were first averaged within assays and then assays within blood specimens. For subjects with multiple blood specimens collected at different times (those in the 3-Monday study and the time course study), levels from only the first specimen (ie, first Monday or Time = 0) were used for regression analyses. (However, all blood specimens were used to investigate the variation of adduct levels at different times.)

The effects of exposure and covariates on albumin adduct levels were investigated with multiple linear regression models (Proc REG of SAS) in which the subject-specific (logged) adduct level was the dependent variable and the logarithm of [1 + (subject-specific geometric mean exposure)] as well as age (in years, continuous variable), sex

(female = 0, male = 1), and smoking status (nonsmoker = 0; smoker = 1) were the independent variables. Models were initially fit to all variables and interaction terms. Then variables were removed in a stepwise fashion, beginning with interaction terms followed by main effects, using the highest *P* value at each step. Final models were constructed in which main effects and interactions were retained at a significance level of $P = 0.10$. If any interaction term was retained, then both main effects were retained regardless of the *P* value. Residual analysis involved visual inspection, examination of studentized residuals to identify possible outliers, and use of Cook distance to identify influential observations. For age-stratified analyses of adduct levels, subjects were first assigned to the highest, medium, or lowest tertile of ages for their population (all workers, male workers, or female workers).

Variance components of albumin adduct levels related to repeated injections (within assays), assays (within blood specimens), specimens (within subjects, 3-Monday study only), and subjects were estimated with Proc NESTED of SAS, which generated ANOVA estimates. Because we assumed an underlying lognormal distribution of adduct levels, the corresponding CVs were estimated as $CV = [\exp(\text{var comp}) - 1]^{1/2}$, where var comp refers to the variance component for the particular source of variation (ie, injection, assay, blood specimen, or subject) obtained from the logged adduct levels.

The correlation of adduct levels with time following exposure (time course study, 0 to 64 hours) was evaluated with Spearman coefficients (Proc CORR of SAS).

Additional Methodologic Analyses to Check on Assumptions

Two sets of analyses were used to check on assumptions (Table A.22 in Appendix A). Although it seems natural to have a 0 exposure point in the study, there may be concerns over the comparability of the control (unexposed) and exposed groups. We took pains to try to obtain a comparable control group, sampling so that they were similar to the exposed group with respect to age, sex, and smoking status and performing semiskilled work at a factory in the same geographic area. Further, all assays were performed in a blinded fashion with exposed and unexposed subjects intermixed. However, given that the exposed and unexposed workers were employed at different factories making different kinds of products, the assumption that the 2 groups were from the same population should be examined. We therefore conducted a set of exposure-response analyses for the more important outcome variables that also included an indicator variable for exposure group, in order to determine the degree to which differences in baseline rates between the exposed and unexposed subjects may have distorted the results. (In Appendix A, the results are shown in the second column of Table A.22 and can be compared to the regression results in Tables A.6 and A.10.) The exposure-response coefficients tended to be smaller when the group indicator variable was included, but this was expected because of shared variance between the 2 coefficients. There was no evidence of marked differences between the results (in Table A.22 and those in Tables A.6 and A.10) that would cause concern regarding disparities between the exposed and unexposed groups.

If the unexposed group were appreciably different from the exposed groups in their baseline risk of the biomarker endpoints under study, one would expect to see this effect most sharply in the exposure-response analyses that examined both linear and quadratic terms in exposure, since a baseline difference would tend to induce nonlinearity (if the curve was linear among the exposed subgroups). We therefore performed more detailed analyses of the linear-quadratic relations of benzene with the biomarker outcomes (Appendix A, Tables A.8 and A.17).

For the albumin adduct and chromosomal aberration analyses (Appendix A, Table A.17), there were no meaningful differences between analyses when the unexposed group was included versus excluded. However, for the blood counts both the linear and quadratic terms for RBCs and neutrophils became less statistically significant. It is not clear how much of these changes was due to loss of statistical power (because excluding the unexposed group produces a smaller sample size) and how much was due to confounding by some unknown variable in the unexposed group.

For the urinary metabolites, we saw no meaningful differences between analyses including or excluding the unexposed group (Appendix A, Table A.8). In addition, we examined whether the statistically significant negative quadratic coefficients (which imply a greater effect per unit dose at low doses than at high doses) for the urinary metabolites might have been due to the choice of scale (ie, we had taken the logarithms of the metabolite variables in almost all cases), which might have induced this curvilinearity. Analyses performed on the raw urinary metabolite variables (Table A.8) also tended to show negative curvilinearity, suggesting it was not primarily a scaling artifact. Again, there was no evidence that including versus excluding the unexposed control group had a substantial impact on the results.

In summary, the bulk of the evidence does not indicate that including the unexposed group in the analyses introduces confounding when analyses have been properly controlled for sex, age, and smoking status.

Another set of methodologic analyses was conducted because the exposure data and some of the outcome

variables were right-skewed. Semiparametric regression analyses based on exposure ranks and outcome-variable ranks (Conover and Iman 1981) were undertaken to see if they corroborated the parametric analyses (last column in Table A.22). In general, the correspondence between the semiparametric results (Table A.22) and the parametric results (Tables A.6 and A.10) was excellent, with substantial disparities for only 3 variables (2 of which—total chromatid aberrations and total aberrations—were highly correlated, so they perhaps should be counted as only a single disparity).

RESULTS

The main characteristics of the exposed and unexposed study subjects are described in Table 1. The members of both groups were almost identical in age and sex distribution. In order to evaluate the confounding effects of smoking on all observed biomarkers, we measured cotinine, a good index of smoking habits, in all urine samples. The results showed that the exposed and unexposed groups did not differ with regard to cotinine levels (Table 1). When we evaluated the results by smoking status, we considered subjects with a mean cotinine level above 100 µg/g creatinine as smokers.

PERSONAL EXPOSURES TO BENZENE, TOLUENE, AND XYLENE

Stability of Benzene in Samplers and Recovery

In order to confirm the stability of benzene in samplers stored at room temperature, 18 personal samplers were divided into 3 groups with 6 samplers each. Then known quantities of benzene were added to the samplers by direct injection. The amounts of benzene added to each sampler for the 3 groups were 1.087, 0.544, and 0.054 mg, respectively. Three samplers from each group were then desorbed with carbon disulfide (CS_2) and analyzed for benzene immediately. The remaining 3 samplers were kept at room temperature and analyzed for benzene 25 days later. There was no significant difference between the benzene recovery measured 25 days later (94.3% ± 3.7% SD) and that measured immediately (91.2% ± 5.4%). The combined recovery was 92.7%.

Field Blanks and Spiked Samples

In total, 130 samples prepared in the field sampling periods (70 blanks and 60 spiked samples) were either analyzed in Beijing or carried back from China to NYU for analysis with the other personal samplers. Benzene, toluene, and xylene were not detected in any blank samples. The recovery measurements from spiked samples prepared in China were similar to those from samples prepared and measured in the NYU laboratory (Table 2), indicating that there was no significant contamination or decay of benzene during collection, storage, and transportation.

Quality Control for GC Analysis

During the entire measurement period, the gas chromatograph was calibrated each day by duplicate injections of 2 laboratory standard solutions (0.055 and 0.218 mg/mL) and of NIST reference standard (200 µg/mg) before sample measurement. In all cases the variation was less than 5% (in accordance with original guidelines for the study), and the samples were then analyzed. During sample analyses, the injection of each of the 2 laboratory standards was repeated after every 10 sample injections. In addition, duplicate injections were conducted for every 10th sample during the day, beginning with sample 5, to make sure that the measurements were reproducible. A paired *t* test for the duplicate measurement of samples indicated no difference between pairs ($P = 0.4608$). The benzene concentrations measured by injection of NIST reference standard were all close enough to the true concentration, and the mean variation ± SD from 200 µg/mL was 1.02% ± 3.69% ($n = 56$); in repeated checks with the laboratory standard solutions (110 injections), the variation was also very small (Table 3).

Table 2. Percent Recovery of Solvents from Field Spiked Samples Measured in Beijing or NYU Laboratories[a]

Solvent	Samples Measured in NYU ($n = 20$)	Samples Measured in Beijing ($n = 40$)
Benzene	90.16±8.92	98.6±7.6
Toluene	89.41±6.04	106.4±9.3
Xylene	95.35±13.75	101.9±7.5

[a] Spiked samples used as quality controls were analyzed with other personal samplers. Values are given as means ± SD.

Table 3. Variations Between Measured and True Concentrations in NIST Reference and Laboratory Standards[a]

Solvent	NIST Reference[b] ($n = 56$)	NYU Laboratory ($n = 110$)	Beijing Laboratory ($n = 52$)
Benzene	1.02±3.69	−0.68±3.11	0.47±6.04
Toluene		−0.56±3.23	0.38±6.98
Xylene		0.53±3.31	1.01±6.86

[a] Values are given as mean percentages ± SD.

[b] Measured at NYU.

Comparison of Benzene Concentrations Measured in NYU and Beijing Laboratories

For the monitoring of personal benzene exposure, duplicate samples were collected from all subjects in phase 1 and for some of the exposure samples in phase 2, with one set analyzed for benzene on site in Beijing, China, and the other set brought back to NYU for analysis. The results of these analyses agreed very well (r^2 = 0.932 for the entire range of exposures, and r^2 = 0.935 for exposures of 5 ppm or lower; Figure 5). Considering that so many factors could affect the results, such as placing the paired samplers in different positions on the subject, differences related to transportation, and differences in length of storage before analysis, the correlation was excellent and we believe the results are valid. Therefore, NYU exposure data are included in the phase 1 analyses presented here as they were used for the related publication (Qu et al 2000). In phase 2 of the study only the exposure samples obtained on the day when biological samples were collected (current-day exposures) included duplicate samples available for analyses at both sites. From the additional 3 personal exposure monitorings before biological sample collection, 10% of samples were duplicated and available for NYU analysis. The complete set of phase 2 exposure samples was analyzed for benzene, toluene, and xylene in Beijing. The two sets of analyses still showed excellent correlation (r^2 = 0.932, not shown). Thus the phase 2 exposure levels reported here are those that were analyzed in Beijing.

Personal Exposure Levels for Benzene, Toluene, and Xylene

Phase 1: Preliminary Sensitivity Assessment Table 4 shows the mean levels of various exposure metrics for all exposed and unexposed groups in phase 1, and for the fractions of the exposed group whose current-day benzene exposure levels were less than 30 ppm or were 30 ppm or higher. The arithmetic mean of the current-day benzene exposure for all exposed subjects was 31.2 ppm; for the group exposed to less than 30 ppm, the mean was 14.3 ppm; and for those exposed to 30 ppm or higher, the mean was 52.7 ppm. The mean benzene exposure levels monitored over 5 weeks fell within the same range as the current-day exposures (Table 4), indicating that the exposure levels were stable from day to day. Compared with the benzene exposure concentrations, toluene and xylene concentrations were considerably lower in these exposed workers.

Area samples using charcoal tubes were also obtained in parallel with personal exposure monitoring on 2 days in the glue factory to determine if the data from the area sampling method currently used in China were comparable to the personal exposure data. The results showed that the levels of benzene and toluene measured in the 2 types of samples were similar (Table 5). This suggested that it was reasonable to estimate individual lifetime cumulative exposure from the historical exposure data that had been obtained by the same area monitoring procedure as is currently used. The mean lifetime cumulative exposure levels in the exposed group are given in Table 4.

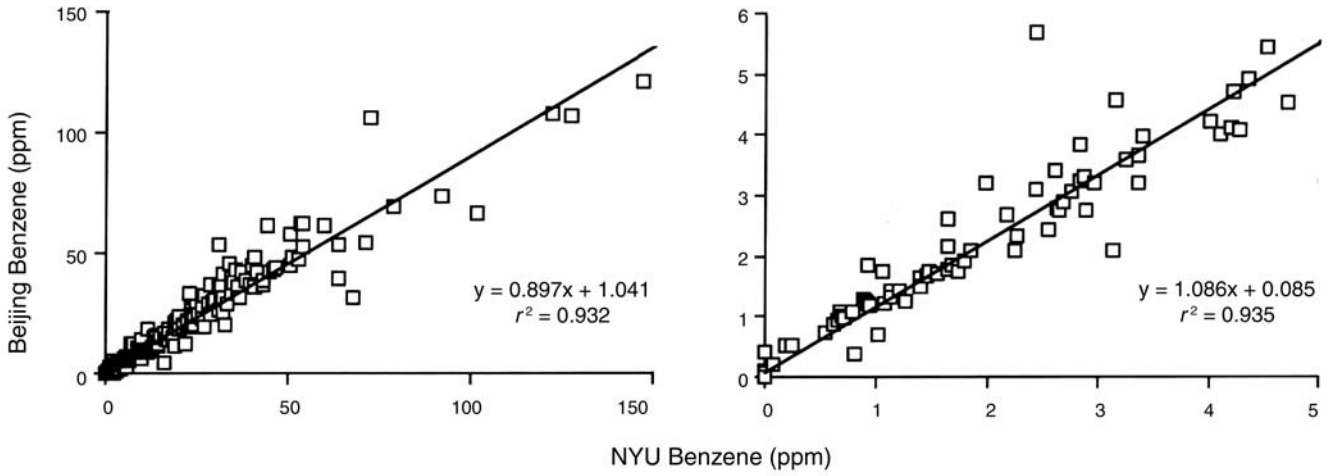

Figure 5. Correlation between benzene concentrations in duplicate personal exposure samples measured at NYU and Beijing laboratories. Left panel shows all data that were measured at both sites (n = 290 sample pairs); right panel shows only low-concentration data (≤ 5 ppm) measured at both sites (n = 132 sample pairs).

Table 4. Personal Exposures to Benzene, Toluene, and Xylene in Phase 1 Subjects

Exposure Metric[a]	Unexposed[b] (n = 25)	Total Exposed (n = 25)	Exposure Levels (ppm)	
			<30 (n = 14)	≥30 (n = 11)
Benzene				
Current-day median	ND	26.2	13.6	45.1
Current-day mean	ND	31.2±25.9	14.3±7.9	52.7±25.0
5-Week mean[c]	ND	35.5±39.5	16.9±8.6	55.7±49.6
5-Week geometric mean[c]	ND	28.7±21.8	15.1±7.9	43.5±22.6
Lifetime cumulative mean (ppm-years)	ND	195±135	196±145	197±130
Toluene				
Current-day mean	ND	2.9±2.1	1.8±1.2	4.2±2.2
Xylene				
Current-day mean	ND	0.11±0.28	0.10±0.18	0.11±0.38

[a] Exposures are given in ppm as means (or geometric mean) ± SD (± GSD). *Current-day means* are the concentrations of benzene, toluene, and xylene exposures measured in personal samples obtained using organic vapor monitors on the same day that biological samples were collected.

[b] ND = not detectable.

[c] *5-Week mean* equals the average of exposure concentrations measured over a 5-week period at intervals of 1 week (average of 5 measurements). For the <30-ppm group, n = 13; for the ≥30 ppm group, n = 12.

Table 5. Levels of Solvents Measured in Both Personal and Area Samples Collected in Glue Factory[a]

Solvent	Area Samples (n = 15)	Personal Samples[b] (n = 34)
Benzene		
Mean±SD	42.9±44.1	36.7±20.8
Range	ND–182.5	3.1–107.2
Toluene		
Mean±SD	5.5±10.2	5.4±4.4
Range	ND–33.8	ND–18.0
Xylene		
Mean±SD	ND	ND

[a] All exposures measured in ppm. ND = not detectable.

[b] Samples were collected from a total of 28 subjects, 6 of whom contributed 2 samples. Eight of the subjects were not recruited during the 2-day monitoring period.

Phase 1: Interindividual and Intraindividual Variability Eleven exposed subjects (6 men and 5 women) were selected from participants in the phase 1 study and monitored for benzene exposures on 3 consecutive Mondays. The mean current-day benzene exposure levels ± SD for the 3 Mondays were 37.0 ± 35.1 ppm, 16.8 ± 9.5 ppm, and 20.5 ± 12.6 ppm, respectively. The exposures to benzene for each individual subject were not constant, and the difference between the highest and lowest levels measured for the same individual varied widely from week to week (range, 4.8 to 118.3 ppm). This made analysis of these data for interindividual and intraindividual variation somewhat complex.

Phase 1: Time Course Study Eleven subjects (4 men and 7 women) were recruited from the glue factory; their mean age ± SD was 34.5 ± 10.4 years. Of these subjects, 7 were among the 25 participants in the phase 1 preliminary sensitivity assessment, from whom samples were collected on Friday, the last workday of the week. Another 4 subjects were not from that group but were also from the glue factory and were monitored for benzene exposure and donated biological samples on the same day. Further collections of blood and urine samples were carried out in the mornings of the following Saturday, Sunday, and Monday (before work) without personal exposure monitoring. The mean exposure concentration of benzene was 25.3 ± 18.2 ppm; toluene, 2.2 ± 1.6 ppm; and xylene, 0.0 ppm.

Phase 2: Examination of Exposure-Response Relation Table 6 shows the mean levels of the different exposure metrics for the total exposed and unexposed groups recruited in phase 2, and for the fractions of the exposed groups whose 4-week mean benzene exposure levels were 5 ppm or less or were greater than 5 ppm. For the overall exposed group, the arithmetic mean 4-week benzene exposure level was 5.2 ppm; for those in the group exposed to 5 ppm or less, it was 2.3 ppm; and for those exposed to greater than 5 ppm, it was 12.1 ppm. Because the exposure distribution was somewhat skewed, as expected, the geometric mean exposures

Table 6. Personal Exposures to Benzene, Toluene, and Xylene in Phase 2 Subjects

Exposure Metric[a]	Unexposed[b] (n = 26)	Total Exposed (n = 105)	Exposure Levels (ppm)	
			>0 to 5 (n = 82)	>5 (n = 23)
Benzene				
Current-day mean	ND	4.6±7.1	1.9±1.4	14.1±10.4
4-Week mean[c]	ND	5.2±7.3	2.3±1.3	12.1±10.3
4-Week geometric mean[c]	ND	4.7±6.7	2.1±1.2	10.7±9.7
Lifetime cumulative mean (ppm-years)	ND	53.8±41.6	42.1±26.1	80.5±56.2
Toluene				
Current-day mean	ND	33.1±32.8	24.9±15.1	51.9±50.4
Xylene				
Current-day mean	ND	0.40±0.42	0.28±0.31	0.65±0.50

[a] Exposures are given in ppm as means (or geometric mean) ± SD (± GSD). *Current-day* means are the concentrations of benzene, toluene, and xylene exposures measured on the same day that biological samples were collected.

[b] ND = not detectable.

[c] *4-Week mean* equals the average of exposure concentrations measured over 4 weeks at intervals of 1 week (average of 4 measurements).

were somewhat lower than the arithmetic mean exposures, especially for the higher exposure group.

Table 6 also shows the estimated lifetime cumulative occupational exposure to benzene, which averaged 53.8 ppm-years in the total exposed group and was higher in the group exposed to greater than 5 ppm (80.5 ppm-years) than in the group exposed to 5 ppm or less (42.1 ppm-years). The correlation between the 4-week mean and lifetime cumulative benzene exposures was moderate (Pearson product moment correlation = 0.44; Spearman rank order correlation = 0.63).

Frequency Distributions of Personal Exposure Levels

Frequency distributions of personal exposure levels to benzene, toluene, and xylene are shown in Figure 6 for all exposed subjects recruited in both phase 1 and phase 2 (levels were measured in the samples collected on the day of biological sample collection). Our original plan was to recruit a substantial number of subjects with benzene exposure levels between 0 and 1 ppm. When exposure monitoring was completed, however, it turned out that many of the subjects had benzene exposure concentrations between 1 and 4 ppm. The exposure distributions tended to be skewed, as shown by large standard deviations and discrepancies between the mean and the median. Compared with benzene exposure levels, toluene levels were high, with a mean of 26.3 ppm and a median of 12.6 ppm. The levels of xylene were low, with a mean of 0.40 ppm and a median of 0.30 ppm. In addition to the current-day exposures, the distribution of lifetime cumulative benzene exposure was calculated (Figure 7). The mean lifetime cumulative benzene exposure ± SD was 81.3 ± 89.3 ppm-years, with a median of 51.2 ppm-years.

BLOOD CELL COUNTS AND DIFFERENTIATION

Both the 4-week mean benzene exposure levels and the lifetime cumulative exposure levels were used while analyzing the association between exposure and blood cell counts because of the long-term effects of benzene on the

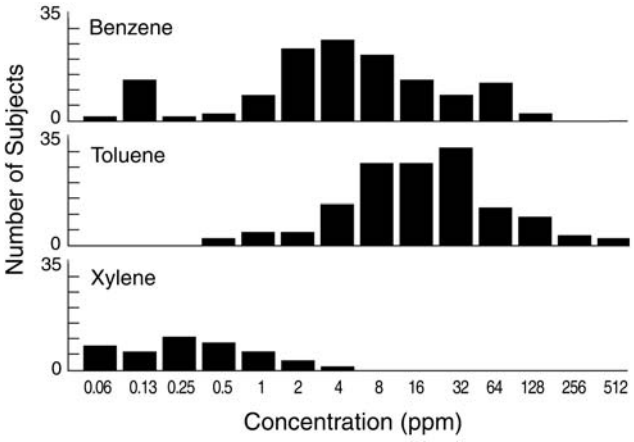

Figure 6. Distribution of current-day benzene, toluene, and xylene exposure levels (ppm) among all exposed subjects (n = 130). Levels were measured in samples obtained on the day of biological sample collection.

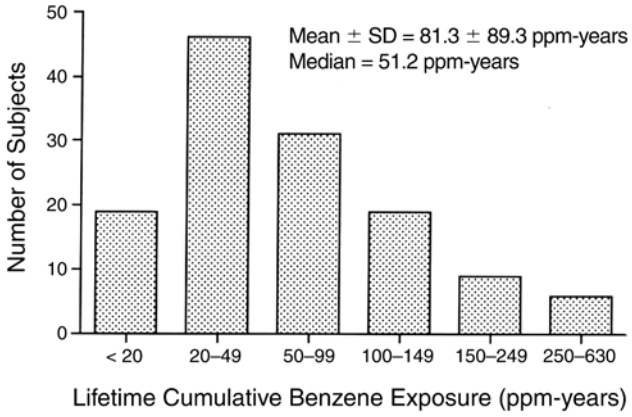

Figure 7. Distribution of lifetime cumulative benzene exposure levels (ppm-years) among all exposed subjects (n = 130). Levels were calculated from personal work histories and related historical exposure records of the factories.

hematopoietic system. Figure 8 shows the overall exposure-response associations for 4-week mean benzene exposure and 6 different blood counts. There were significant exposure-dependent decreases in RBCs, WBCs, and neutrophils. Somewhat surprisingly, in this analysis there was no clear benzene effect on lymphocytes, nor was there any effect on platelets. Furthermore, there was no exposure effect on monocytes, eosinophils, basophils, band cells, or atypical lymphocytes (Appendix A, Table A.9). Other endpoints measured, such as metamyelocytes, microcytosis, macrocytosis, poikilocytosis, hypochromia, and polychromasia, were too rare to quantify meaningfully.

Regression analyses of the blood cell count data on 4-week mean benzene exposures were also conducted. Significant negative associations were found between benzene exposure and RBCs, WBCs, neutrophils, and atypical lymphocytes (Appendix B, Table B.1). In addition, once adjustments were made for potential confounders, there were weak negative associations between benzene exposure and decreased lymphocyte and monocyte counts (Table B.1). A further analysis (not shown) of confounding variables in relation to lymphocyte counts suggested that smoking status may have masked the association between benzene and lymphocyte count in the unadjusted analyses.

To determine whether the associations between benzene exposure and various blood cell counts were linear or curvilinear, further analysis was done by fitting models that included both exposure (linear) and exposure-squared terms for benzene exposure. The results did not reveal any indication of quadratic curvature in the exposure response for any of the blood counts (analyses not shown). Analyses were performed to examine blood counts at low levels of benzene exposure (≤ 1 ppm). As it turned out, for 4-week mean benzene exposures, all the subjects exposed to concentrations of 1 ppm or lower were actually exposed to less than 0.25 ppm, so it was not possible to look at gradations of exposure in this range. When data for unexposed subjects and subjects with 4-week mean benzene exposures of 0.5 ppm or lower were compared (Table 7), there were statistically significant depressions in RBCs, WBCs, and neutrophils in the subjects with low benzene exposures. Multiple regression analyses showed that these associations remained after control of confounding variables.

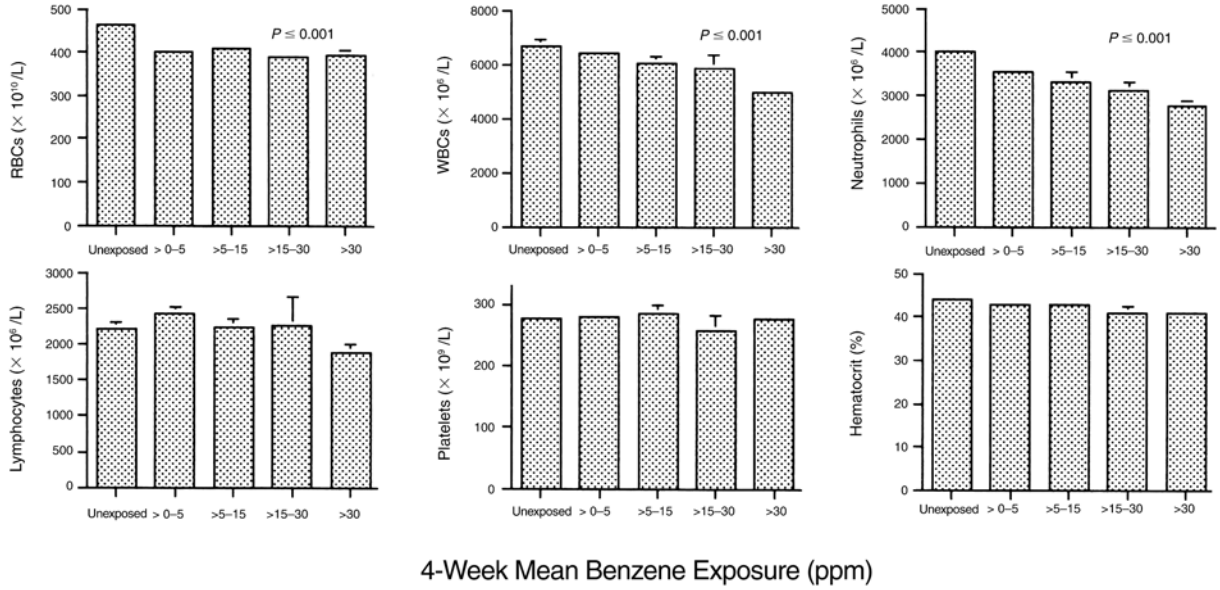

Figure 8. Blood cell counts among all subjects (n = 181) grouped according to 4-week mean benzene exposure (ppm). Error bars represent the standard errors of the means. There were significant exposure-dependent decreases in RBCs, WBCs, and neutrophils ($P \leq 0.001$). Test for exposure-response trend was based on ANOVA linear contrast.

Figure 9 shows the means of 6 of the blood cell counts by lifetime cumulative benzene exposure levels (ppm-years). RBCs, WBCs, and neutrophils decreased significantly in relation to increasing cumulative benzene exposure. However, for RBCs the association seemed to be primarily a difference between the unexposed and exposed groups, with little gradation according to the amount of cumulative exposure, reducing confidence in the association.

Table 7. Differences in Blood Cell Counts Between Unexposed Subjects and Subjects with 4-Week Mean Benzene Exposures of 0.5 ppm or Lower[a]

Variable	Unexposed	Exposed (>0 to 0.5 ppm)	Significant *P* value[b]
Number of subjects	51	16	NA
Female (%)	53	100	NA
Smoker (%)[c]	31	0	NA
Age (years)	33.3±7.4	36.2±3.2	NA
4-Week mean benzene exposure	0.004±0.003	0.14±0.04	—
Red blood cells ($\times 10^{10}$/L)	463±52	393±49	0.0006
Hematocrit	44.2±5.3	43.1±2.6	—
Platelets ($\times 10^{9}$/L)	277±43	286±71	—
White blood cells ($\times 10^{6}$/L)	6671±1502	5700±1226	0.02
Lymphocytes ($\times 10^{6}$/L)	2205±789	2015±450	—
Neutrophils ($\times 10^{6}$/L)	4006±1108	3254±901	0.02
Monocytes ($\times 10^{6}$/L)	267±139	251±108	—
Eosinophils ($\times 10^{6}$/L)	145±162	136±133	—
Basophils ($\times 10^{6}$/L)	9.3±19.3	10.1±14.6	—
Band cells	32.6±44.1	33.5±40.3	—
Atypical lymphocytes	0.10±0.22	0.04±0.11	—

[a] Values are given as means ± SD of the raw variables; but for the monocytes, eosinophils, and basophils, the statistical tests were performed on the log-transformed data.

[b] Least-squares regression analysis controlling for sex, age, smoking, cotinine level, toluene exposure, and year (phase) of study. NA = not applicable. Dashes indicate $P \geq 0.05$.

[c] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

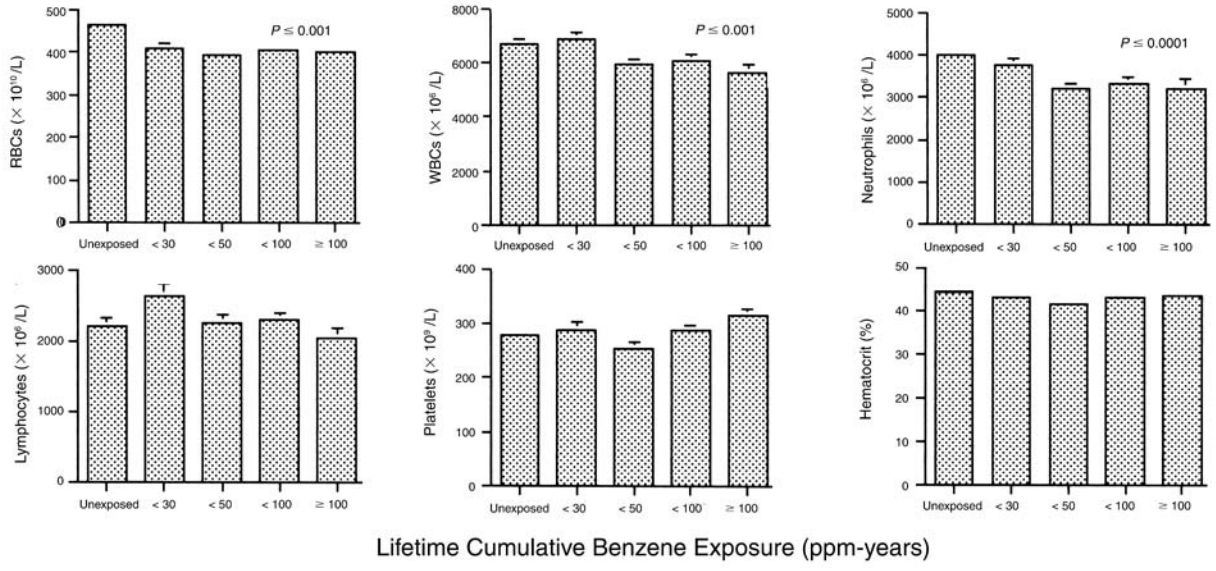

Figure 9. Blood cell counts among all subjects (n = 181) grouped according to lifetime cumulative benzene exposure (ppm-years). The error bars represent the standard errors of the means. There were significant exposure-dependent decreases in neutrophils ($P \leq 0.0001$) and in RBCs and WBCs ($P \leq 0.001$). Test for exposure-response trend was based on ANOVA linear contrast.

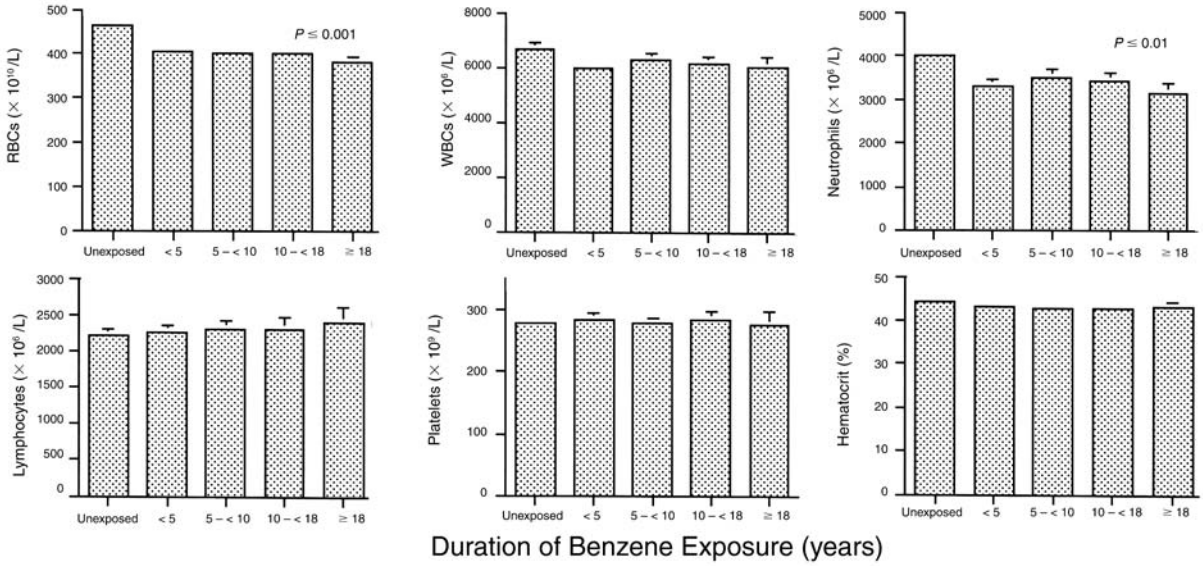

Figure 10. Blood cell counts among all subjects (n = 181) grouped according to duration of benzene exposure (years). The error bars represent the standard errors of the means. There were significant exposure-dependent decreases in RBCs ($P \leq 0.001$) and neutrophils ($P \leq 0.01$). Test for exposure-response trend was based on ANOVA linear contrast.

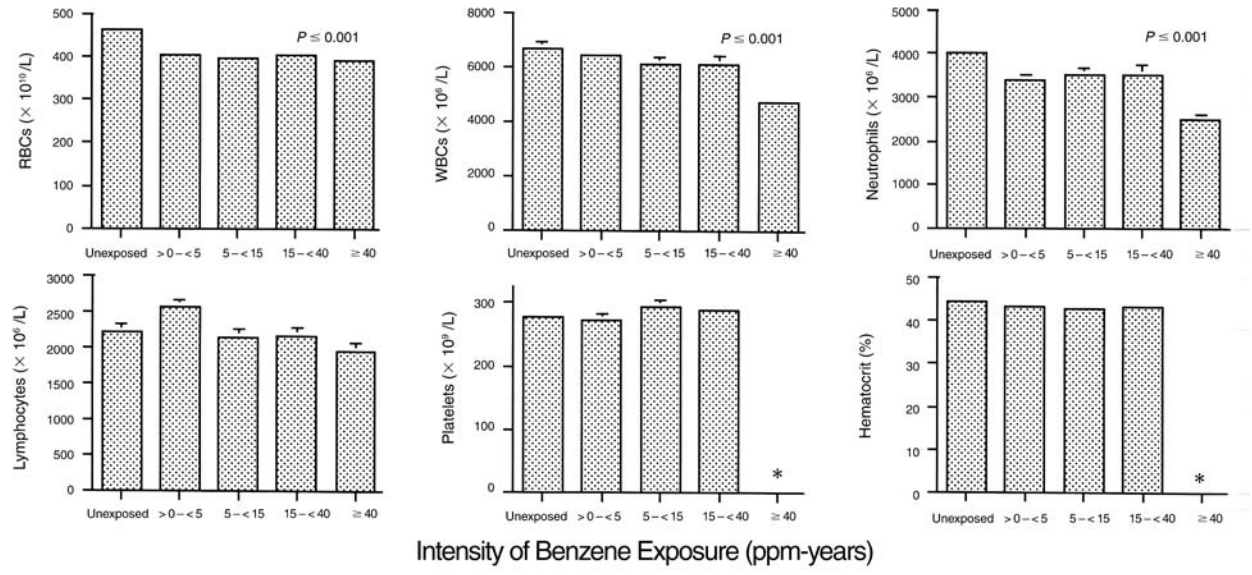

Figure 11. Blood cell counts among all subjects (n = 181) grouped according to intensity of benzene exposure (ppm-years). The error bars represent the standard errors of the means. There were significant exposure-dependent decreases in RBCs, WBCs, and neutrophils ($P \leq 0.001$). Test for exposure-response trend was based on ANOVA linear contrast.

* No data available because all subjects in this group are from phase 1 of the study.

Regression analyses by cumulative benzene exposure were conducted in order to adjust for possible confounding variables (Appendix B, Table B.2). The adjusted analyses showed strong inverse associations of cumulative benzene exposure with RBCs, WBCs, neutrophils, and monocytes, as well as weaker, but statistically significant, associations for lymphocytes and eosinophils. There was also a weak positive association of benzene exposure with platelet counts, which may have been a chance finding.

An initial analysis was conducted to determine the relative contributions to the observed cumulative effects on blood cell counts of benzene exposure duration (Figure 10) and intensity, given as estimated mean exposure concentration per year (Figure 11). Only RBC and neutrophil levels were significantly associated with exposure duration, while RBC, WBC, and neutrophil levels, as well as eosinophil levels (not shown), were significantly associated with exposure intensity.

To further compare the contributions of duration and intensity of benzene exposures to the effects on blood cell counts in relation to long-term cumulative exposures, an exposure-response regression analysis was conducted with both duration and intensity in the model. In the results of this analysis, benzene exposure intensity predicted depression in RBC, WBC, lymphocyte, neutrophil, monocyte, and eosinophil counts, while exposure duration showed a weak association only for neutrophils (Appendix B, Table B.3). Hence, benzene exposure intensity appeared much more predictive of bone marrow depression than duration of exposure.

VALIDATION OF URINARY METABOLITES

Reliability for Analyses of Urinary Metabolites

The CVs for 6 urinary metabolites were determined in the 10 pooled samples prepared for quality control both in phase 1 and in phase 2 (Table 8). The pooled samples prepared for aliquots differed for phase 1 and phase 2, so the 2 sets of data could not be combined for analysis and are presented here separately. The values of *t,t*-MA and *S*-PMA for 1 sample in the phase 1 study were much lower than those for the 9 other samples (less than 2 SD below the mean). Table 8 presents the results after exclusion of this sample. The CVs for *S*-PMA, *t,t*-MA HQ, CAT, and BT ranged from 11.6% to 23.8%, but the CVs for phenol were higher. It is important to note that the variability measurement being described here reflects not only duplicate measurements performed in a laboratory run, as are commonly used to assess reliability, but also variability across runs in the laboratory, so it reflects day-to-day variability as well.

Phase 1: Preliminary Sensitivity Assessment

The concentrations of the urinary biomarkers *S*-PMA, *t,t*-MA, HQ, CAT, BT, and phenol were all first standardized by the concentration of creatinine in urine before statistical analyses. These urinary metabolites of benzene were measured as continuous variables but proved to be right-skewed. A logarithmic transformation [$\ln(x + 1)$] was therefore applied to these variables for statistical analysis.

The metabolites of benzene are expected to have a fast clearance from urine, so the current-day benzene exposure levels (those on the day when urine samples were collected) were primarily used to correlate with corresponding changes in the urinary metabolites.

Table 8. Replicabilities of Urinary Metabolites Measured in Pooled Quality Control Samples[a]

Urinary Metabolite[c]	Phase 1		Phase 2[b]	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
S-PMA	379±53	14.1	50.8±8.6	16.9
t,t-MA	11.7±2.8	23.8	2.11±0.25	11.6
HQ	25.8±4.1	15.7	NA	NA
CAT	11.1±1.6	14.5	NA	NA
BT	10.4±1.8	17.4	NA	NA
Phenol	141±103	72.8	6.4±4.7	65.4

[a] Based on measurements of 10 pooled samples performed at random in various runs after excluding 1 value below the lower 95% confidence limit for *S*-PMA and *t,t*-MA in phase 1.

[b] NA = not applicable (not analyzed in phase 2).

[c] All metabolites were measured in mg/g creatinine except for *S*-PMA in µg/g.

Table 9. Urinary Metabolite Levels Measured in Unexposed Subjects Grouped According to Sex and Cotinine Level, Phase 1 Only[a]

Urinary Metabolite	Sex		Cotinine Level (μg/g creatinine)	
	Female (n = 13)	Male (n = 12)	≤100 (n = 17)	>100 (n = 8)[b]
S-PMA				
Before work	0.93 ± 1.78	1.58 ± 2.75	1.02 ± 1.61	1.71 ± 3.38
After work	0.37 ± 0.67	1.44 ± 1.28[c]	0.51 ± 0.81	1.67 ± 1.36[c]
t,t-MA				
Before work	0.69 ± 0.94	0.26 ± 0.20	0.60 ± 0.84	0.24 ± 0.21
After work	0.34 ± 0.35	0.28 ± 0.25	0.33 ± 0.31	0.27 ± 0.29
HQ				
Before work	4.20 ± 2.52	4.49 ± 3.44	4.64 ± 2.93	3.70 ± 3.66
After work	4.78 ± 5.20	3.52 ± 3.34	4.27 ± 4.62	3.98 ± 3.80
CAT				
Before work	2.43 ± 2.35	3.29 ± 1.29	2.84 ± 2.20	2.85 ± 1.39
After work	2.28 ± 1.74	2.65 ± 1.62	2.28 ± 1.56	2.83 ± 1.80
Phenol				
Before work	17.19 ± 18.67	15.51 ± 15.45	17.12 ± 17.21	14.83 ± 15.16
After work	15.00 ± 14.21	8.45 ± 7.14	12.79 ± 13.58	10.67 ± 7.23

[a] All metabolite levels are presented as arithmetic means ± SD. Metabolites were measured in mg/g creatinine except for *S*-PMA in μg/g.

[b] All subjects who had cotinine levels >100 μg/g creatinine were male.

[c] $P < 0.05$ (male vs female; cotinine ≤100 vs cotinine >100), by t tests.

Urinary Metabolites in Unexposed Subjects The background level of each urinary metabolite was determined in the 25 unexposed subjects in phase 1 (Table 9). In general, the background levels of *S*-PMA and *t,t*-MA were very low compared with those of HQ, CAT, and phenol, suggesting that contributions to their production from sources other than benzene were minimal. In particular, the levels of *S*-PMA in most of the subjects were below the limit of detection. Among the 25 unexposed subjects, 8 were smokers according to their cotinine levels (> 100 μg/g creatinine), and all happened to be male. Except for after-work levels of *S*-PMA, no significant difference in urinary metabolite levels was detected either between smokers and nonsmokers or between male and female workers.

Urinary Metabolites in Exposed Subjects With the exception of BT, the levels of benzene metabolites measured in urine collected from exposed subjects at the end of each work shift were significantly higher than those measured in unexposed subjects (Figure 12; $P < 0.0001$). The correlations with log-transformed current-day benzene exposure were 0.96 for *S*-PMA, 0.93 for *t,t*-MA, 0.89 for HQ, 0.89 for CAT, and 0.75 for phenol (Table 10). In addition, large increases from before-work to after-work levels of *S*-PMA, *t,t*-MA, HQ, CAT, and phenol were detected in the exposed group ($P < 0.001$). The correlations of these increases with log-transformed benzene exposure were 0.69 for *S*-PMA, 0.66 for *t,t*-MA, 0.82 for HQ, 0.83 for

Table 10. Pearson Product Moment Correlations of Urinary Metabolite Levels with Current-Day Benzene Exposure for Exposed Subjects (n = 25), Phase 1 Only

Urinary Metabolite[a]	Raw Data	Log-Transformed	Rank Order
S-PMA			
After work	0.72	0.96	0.88
After − before	0.53	0.69	0.69
t,t-MA			
After work	0.67	0.93	0.84
After − before	0.42	0.66	0.68
HQ			
After work	0.71	0.89	0.87
After − before	0.64	0.82	0.85
CAT			
After work	0.72	0.89	0.88
After − before	0.66	0.83	0.83
Phenol			
After work	0.69	0.75	0.74
After − before	0.63	0.57	0.58

[a] The raw metabolite data for after-work and after-work − before-work levels were all log-transformed.

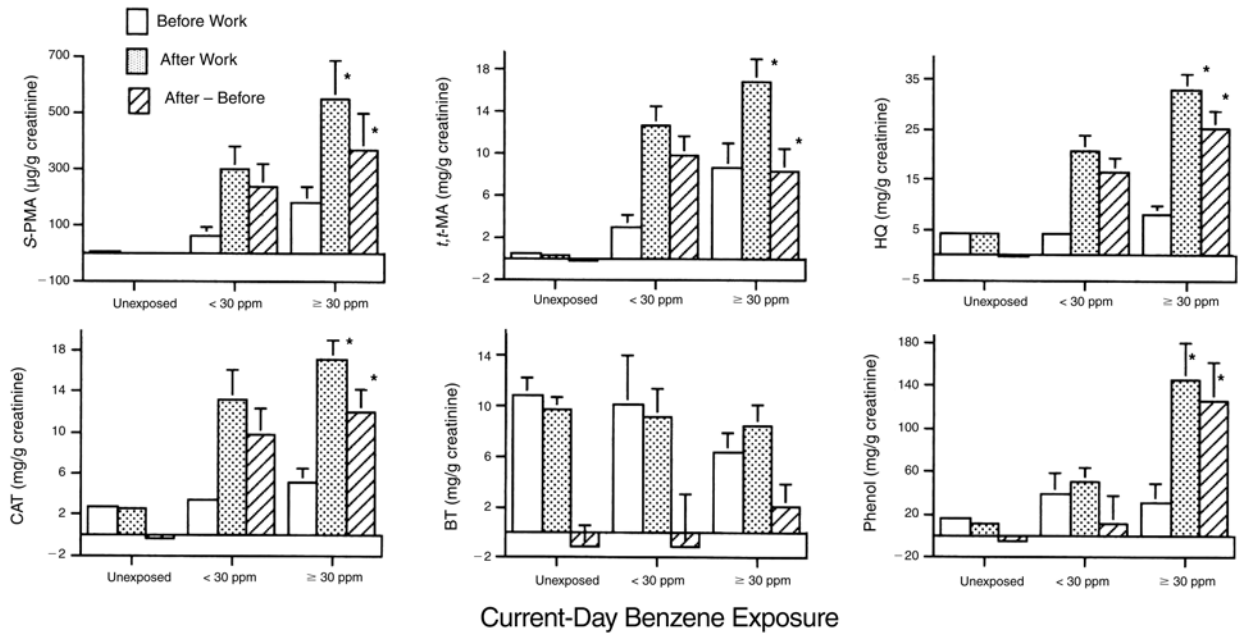

Figure 12. Levels of urinary metabolites among phase 1 subjects grouped according to the current-day benzene exposure: unexposed (n = 25); < 30 ppm (n = 14), and ≥ 30 ppm (n = 11). Exposure levels were determined before work and after work, and the difference was calculated.
* $P \leq 0.0001$, by ANOVA trend tests to compare the 3 exposure groups.

CAT, and 0.57 for phenol (Table 10). Analyses of these benzene metabolite variables versus current-day benzene exposure levels (as a continuous variable), controlling for age, sex, and smoking status as possible confounders and then determining whether other exposure measurements added predictiveness, continued to show strong associations. None of the variables showed any confounding by age, sex, or smoking, but HQ, CAT, and *S*-PMA showed an additional association with toluene exposure.

Furthermore, analyses comparing the lower exposure group, exposed to less than 30 ppm benzene, and the unexposed group still revealed strong associations between exposure levels and HQ, CAT, *S*-PMA, *t,t*-MA, and phenol, although the difference between the groups was not as great for phenol ($P = 0.02$) as for the other metabolites (all $P < 0.001$; Figure 12), and no significant difference was found for BT.

The correlations among urinary metabolites for samples collected at the end of the workday were high with the exception of BT. For example, the Pearson product moment correlations of log-transformed HQ data with log-transformed data for the other metabolites were 0.87 for *S*-PMA, 0.88 for *t,t*-MA, 0.89 for CAT, and 0.75 for phenol (Table 11). Other correlations were equal to or greater than 0.80: ie, CAT with *S*-PMA (0.89), CAT with *t,t*-MA (0.85), CAT with phenol (0.80), and *S*-PMA with *t,t*-MA (0.94). Spearman rank order correlations were also examined to see whether skewness in the variables might have greatly influenced the Pearson correlations, but the Spearman rank order correlations were uniformly similar in magnitude to the parametric correlations.

When the difference variables (after-work values minus before-work values) were examined, the correlations among them were lower than they had been for the after-work variables (Table 11). The lower correlations probably occurred for two reasons. First, a difference score has a greater random error component than a simple variable (ie, the variance of the difference score is the sum of the variances for the 2 variables constituting the difference score), which would tend to reduce the correlation. Second, even though most of the measurements were made on Monday so as to reduce the amount of metabolite attributable to previous days' benzene exposure (ie, by allowing 2 intervening weekend days without exposure), there nevertheless was carryover to varying degrees for several metabolite measures. In spite of these problems, some Pearson correlations among the difference scores were above 0.70 (Table 11): HQ with CAT (0.81), HQ with *S*-PMA (0.71), and *S*-PMA with *t,t*-MA (0.75).

Phase 1: Interindividual and Intraindividual Variability

Within-worker variability over time with regard to benzene metabolite excretion was analyzed with data gathered on 3 consecutive Mondays from 11 subjects. Mondays were chosen so that there would be less carryover from

Table 11. Pearson Product Moment Correlations Among Mean Urinary Metabolite Levels in Benzene-Exposed Subjects (n = 25), Phase 1 Only[a]

Urinary Metabolite	*S*-PMA		*t,t*-MA		HQ	CAT		Phenol	
	After	After − Before	After	After − Before	After − Before	After	After − Before	After	After − Before
S-PMA									
After	—	0.70	0.94	0.64	0.76	0.89	0.79	0.74	0.31
After − Before	0.70	—	0.65	0.75	0.71	0.64	0.65	0.53	0.61
t,t-MA									
After	0.94	0.65	—	0.73	0.76	0.85	0.75	0.68	0.49
After − Before	0.64	0.75	0.73	—	0.69	0.60	0.67	0.38	0.39
HQ									
After	0.87	0.62	0.88	0.61	0.86	0.89	0.79	0.75	0.48
After − Before	0.76	0.71	0.76	0.69	—	0.74	0.81	0.62	0.45
CAT									
After	0.89	0.64	0.85	0.60	0.74	—	0.83	0.80	0.58
After − Before	0.79	0.65	0.75	0.67	0.81	0.83	—	0.68	0.54
Phenol									
After	0.74	0.53	0.68	0.38	0.62	0.80	0.68	—	0.75
After − Before	0.31	0.61	0.49	0.39	0.45	0.58	0.54	0.75	—

[a] All variables were log-transformed.

previous workdays. The analysis of measurement consistency within individuals was indexed by CV. The analyses in this particular set were unconventional in that they had to be based on a mixed model analysis of covariance, rather than the usual simple standard deviation, because of the fluctuation in benzene exposure levels for individuals from one Monday to the next. In effect, this analysis asks whether there is a consistency in metabolite levels within individuals over time when benzene exposure levels are held constant. This analysis was conducted for each urinary metabolite (Appendix B, Table B.4). In view of the strong associations between most of the urinary metabolites and benzene exposure levels, it was expected that intraindividual variability would be rather small and the intraclass correlations would be high. However, this did not prove to be the case for some measures (Table B.5). The associations were generally not very strong for the measured metabolite levels after work, but were appreciably stronger for the differences between after-work and before-work metabolite levels.

Phase 1: Time Course Study

Two types of data collected from this study were used to estimate the residence time of the urinary metabolites in the body after cessation of benzene exposure. The data generated from a time course study in phase 1 were the basis for estimating the elimination half-lives of benzene metabolites, whereas the data from morning (before-work) urine samples collected on Monday and Friday were analyzed to estimate any accumulation of metabolites attributable to previous days' exposure.

Eleven benzene-exposed subjects were recruited for the time course study. They were first asked to provide urine samples in the morning before work on Friday and then were repeatedly monitored for benzene exposure. At the end of the work shift on Friday they again were asked to provide urine samples (the 0 time point). Additional urine samples were collected on the mornings of the following Saturday (16 hours), Sunday (40 hours), and Monday (64 hours). Because of logistic difficulties for the workers, the 16-hour sample was collected from only 6 of the subjects, and the 40-hour sample from the other 5 subjects. Samples were collected from all 11 workers at the 0 and 64-hour time points. Thus concentrations of urinary metabolites were measured in samples collected at 0, 16, 40, and 64 hours, beginning at the end of the Friday shift. There was no known additional exposure of the workers to benzene over the 64 hours. Measurements in the unexposed control population revealed a baseline urinary concentration for each of the metabolites in the absence of work exposure (Table 12). The mean concentration in unexposed subjects ranged from less than 1% (*S*-PMA) to 23% (CAT) of the mean concentration measured in exposed subjects at the end of a Friday shift, and from 5% (*S*-PMA) to 102% (HQ) of the mean concentration at 64 hours. Thus it is

Table 12. Time Course Study: Mean Urinary Metabolite Levels in Benzene-Exposed Subjects and Background Levels in Unexposed Subjects, Phase 1 Only[a]

		Time Course of Benzene Metabolism			
Urinary Metabolite	Unexposed (n = 25)	0 Hours (n = 11)	16 Hours (n = 6)	40 Hours (n = 5)	64 Hours (n = 11)
S-PMA	0.88 ± 0.23	409 ± 163	297 ± 226	46.0 ± 4.6	17.2 ± 3.8
t,t-MA	0.31 ± 0.06	13.3 ± 2.7	10.6 ± 10.2	1.58 ± 0.18	1.03 ± 0.18
HQ	4.34 ± 0.60	22.2 ± 5.0	7.65 ± 0.98	6.17 ± 0.99	4.25 ± 1.42
CAT	2.84 ± 0.58	12.3 ± 2.5	3.52 ± 0.63	4.51 ± 1.04	3.04 ± 1.01
Phenol	11.9 ± 2.4	98.3 ± 41.7	25.0 ± 12.6	21.6 ± 7.5	15.9 ± 4.1

[a] Values are presented as arithmetic means ± SD. All metabolites were measured in mg/g creatinine except for *S*-PMA in µg/g.

clear that a source other than benzene exposure was contributing to the metabolite levels.

To calculate the clearance time for benzene, as represented by each metabolite, it was necessary to account for the additional source responsible for the baseline concentration of each metabolite. This was accomplished by assuming a constant source for each, as well as first-order clearance to the urine, leading to a constant background urinary concentration (when corrected for creatinine content). Since it was not possible from these data to obtain an appropriate estimate of the baseline level in each individual worker, the value was assumed to be equal to the mean concentration measured in the control population. Also, not all of the same workers were measured at 16, 40, and 64 hours, so the mean values for the group were taken as the best estimates of the concentration at these times. Subtracting the control value from the mean value measured for each metabolite at each time point allowed estimation of the rate at which clearance of the workplace benzene exposure took place over the weekend. Values of the net urinary concentration for each metabolite were fitted to an equation of the form

$$C = C_0 \, e^{-(at)}$$

where C = net concentration in urine at time t, C_0 is the net concentration at the end of the Friday shift, a is a decay constant equal to $0.693/t_{1/2}$ where $t_{1/2}$ represents the half-life of the metabolite in the urine. The correlations r for the fit were 0.946, 0.919, 0.883, and 0.930 for *S*-PMA, *t,t*-MA, CAT, and phenol, respectively. Half-life estimates for the same metabolites were 12.8, 13.7, 15.0, and 16.3 hours, respectively. Since the net mean HQ concentration at 64 hours was nearly 0, the curve fit utilized only 3 time points, giving a $t_{1/2}$ estimate of 12.7 hours and r = 0.966. The range of half-lives suggested that a mean ± SD of $t_{1/2}$ = 14 ± 1 hours could be applied to all of the measured metabolites, although it should be noted that the variability among subjects was considerable (see Table 12).

Since clearance times were approximately the same for all of the metabolites, the metabolite that was most elevated as compared with the background (unexposed control) level would be the most reasonable for detecting recent exposure. Thus *S*-PMA, followed by *t,t*-MA, could provide sensitive markers.

Furthermore, in the phase 1 preliminary sensitivity assessment study, high background levels of urinary metabolites were also observed in samples collected from 25 exposed subjects before work. These may have reflected the relatively slow clearance or accumulation of urinary metabolites from the previous 1 or 2 days of exposure. This hypothesis was confirmed by further analysis of these data. Among these urine samples, 13 were collected from workers on Monday morning and 12 on Friday morning. The major difference between the 2 sets of samples was that subjects who donated urine samples on Monday morning had had no previous exposures for at least 2 days before providing urine, whereas subjects providing samples on Friday morning had had 4 days of continuous exposure before sampling. The concentrations of all metabolites except BT in these samples are given in Table 13. As expected, the levels of *S*-PMA, *t,t*-MA, and phenol in Friday morning samples were significantly higher than those in Monday morning samples, indicating a substantial effect of previous-days' benzene exposure on the levels of *S*-PMA, *t,t*-MA, and phenol in urine. Similar results were obtained in the time course study, in which samples were collected from the same 11 workers on Monday morning and on Friday morning (Table 13).

Phase 2: Examination of Exposure-Response Relation

Urinary Metabolites in Unexposed Subjects A total of 51 unexposed subjects were considered in phase 2 of this study, 25 subjects from the preliminary sensitivity assessment in phase 1 and 26 more recruited during phase 2. Among them, 27 were women, and 16 were considered to be smokers because they had cotinine levels greater than 100 µg/g creatinine. The mean concentration of urinary cotinine in unexposed subjects was 560 ± 1125 µg/g creatinine. In phase 1 of the study, after-work levels of urinary *S*-PMA were significantly higher in smokers than in nonsmokers (see Table 9); however, when phase 1 and phase 2 data were combined, this difference was not significant. In phase 2, as in phase 1, there were no significant differences in urinary *t,t*-MA and phenol, either between smokers and nonsmokers or between male and female workers (Table 14). Data on HQ, CAT, and BT levels were not available because they were not measured in phase 2 of the study.

Table 13. Levels of Urinary Metabolites Collected from Benzene-Exposed Subjects on Monday and Friday Mornings Before Work, Phase 1 Only

Urinary Metabolite[a]	Preliminary Sensitivity Assessment[b]		Time Course Study[c]	
	Monday (n = 13)	Friday (n = 12)	Monday (n = 11)	Friday (n = 11)
S-PMA	50.1±76.5	194±187[d]	17.2±11.9	233±175[f]
t,t-MA	3.2±4.4	8.3±8.6[e]	1.0±0.6	10.1±8.4[g]
HQ	5.6±5.8	6.4±3.7	4.3±1.6	7.3±3.4[e]
CAT	4.5±4.7	4.1±1.6	3.0±1.0	3.2±2.2
Phenol	10.2±10.6	61.1±88.9[d]	15.8±13.9	80.9±88.4[e]

[a] All metabolites were measured in mg/g creatinine except for *S*-PMA in µg/g. Values are presented as arithmetic means ± SD. Paired *t* tests were used to compare temporal data.

[b] Samples were collected from different subjects on Monday and Friday.

[c] Samples were collected from the same 11 subjects on Monday and Friday.

[d] Significantly different from Monday levels ($P < 0.01$).

[e] Significantly different from Monday levels ($P < 0.05$).

[f] Significantly different from Monday levels ($P < 0.001$).

[g] Significantly different from Monday levels ($P < 0.005$).

Table 14. Levels of Urinary Metabolites Measured in All Unexposed Subjects Grouped According to Sex and Cotinine Level[a]

Urinary Metabolite[b]	Sex		Cotinine Level (µg/g creatinine)	
	Female (n = 27)	Male (n = 24)	≤100 (n = 35)	>100 (n = 16)[c]
S-PMA				
Before work	2.17±5.60	1.40±2.10	1.83±4.95	1.74±2.48
After work	1.67±2.01	2.10±1.68	1.63±1.87	2.40±1.77
t,t-MA				
Before work	0.47±0.76	0.20±0.19	0.40±0.68	0.22±0.21
After work	0.30±0.33	0.22±0.19	0.28±0.30	0.22±0.21
Phenol				
Before work	8.72±15.23	15.07±26.19	9.30±14.39	16.99±31.20
After work	8.37±12.18	5.15±6.50	7.30±11.00	5.88±7.40

[a] Values are presented as arithmetic means ±SD.

[b] All metabolites were measured in mg/g creatinine except for *S*-PMA in µg/g.

[c] All subjects were male workers.

Urinary Metabolites in Exposed Subjects When the exposed and unexposed groups were compared with regard to *S*-PMS, *t,t*-MA, and phenol levels, the differences associated with benzene exposure were obvious (Figure 13). The urinary metabolite data were then broken down into 5 groups according to subjects' levels of benzene exposure on the day of urine collection. Even at exposure levels of 1 ppm and lower, large increases were observed in the samples collected after work compared with levels determined before work for *S*-PMA in particular (Figure 14), and also for *t,t*-MA (Figure 15). However, 4 workers had higher *S*-PMA levels in the morning before work than after work (reflected in the negative values in Figure 14 in the subgroup with benzene exposure ≤ 1 ppm). In contrast, phenol levels increased only with benzene exposure levels of 5 ppm or greater (Figure 16). In virtually all cases, the exposure-response trend was positive and statistically significant over the whole range of exposures.

For the 130 exposed subjects, current-day benzene exposure was plotted against the after-work *S*-PMA concentration (Figure 17) and *t,t*-MA concentration (Figure 18); the data were all log-transformed. The exposure-response trend was not adjusted for any possible confounding variables; however, over this broad range of exposures, a significant correlation was established between *S*-PMA and benzene exposure ($r = 0.72$, $n = 130$, $P < 0.0001$) and between *t,t*-MA and benzene exposure ($r = 0.74$, $P < 0.0001$).

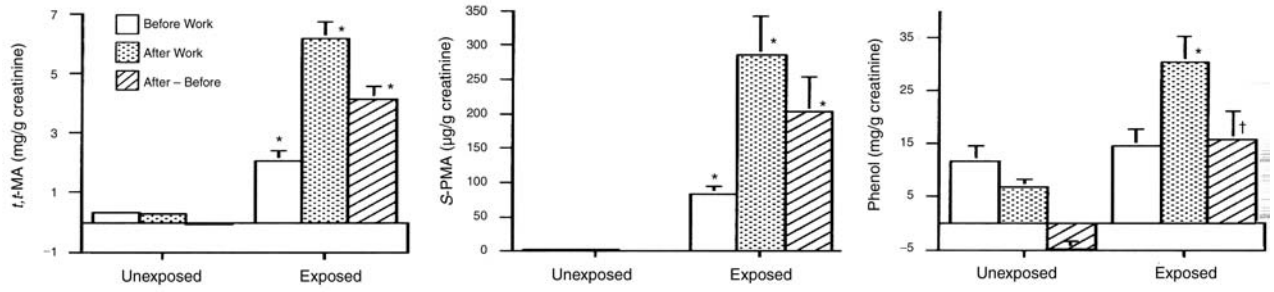

Figure 13. Differences in levels of urinary metabolites between all exposed subjects ($n = 130$) and unexposed subjects ($n = 51$). The means and standard errors plotted are raw variables, but *t* tests to compare values between the groups were performed on the log-transformed data. * $P \leq 0.001$; † $P \leq 0.05$.

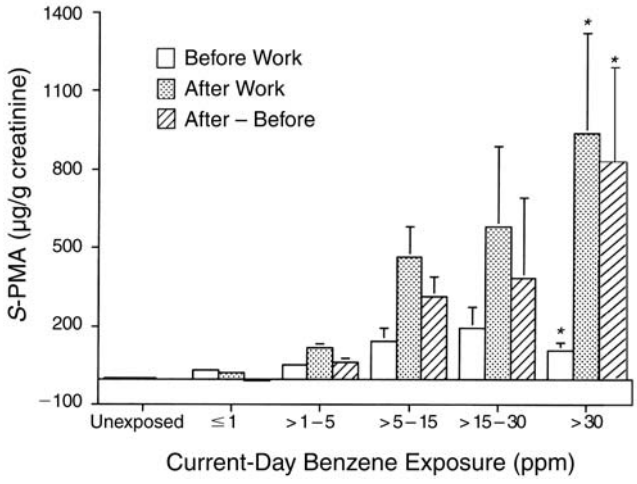

Figure 14. Urinary *S*-PMA levels (means ± SE) among all unexposed subjects ($n = 51$) and exposed subjects ($n = 130$) grouped according to the current-day benzene exposure. * $P \leq 0.001$, test for exposure-response trend based on an ANOVA linear contrast.

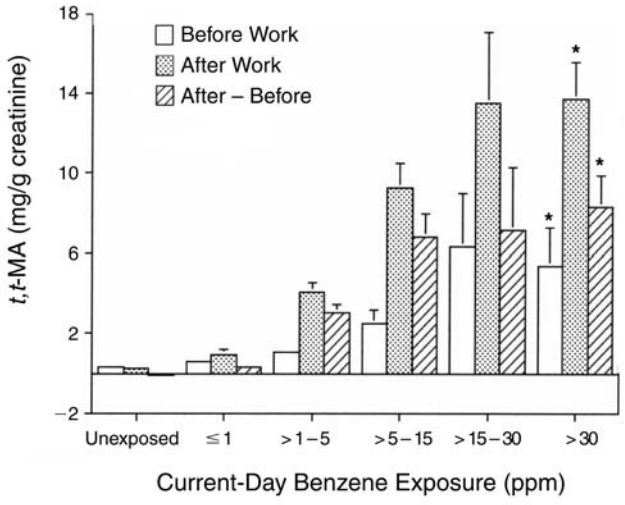

Figure 15. Urinary *t,t*-MA levels (means ± SE) among all unexposed subjects ($n = 51$) and exposed subjects ($n = 130$) grouped according to the current-day benzene exposure. * $P \leq 0.001$, test for exposure-response trend based on an ANOVA linear contrast.

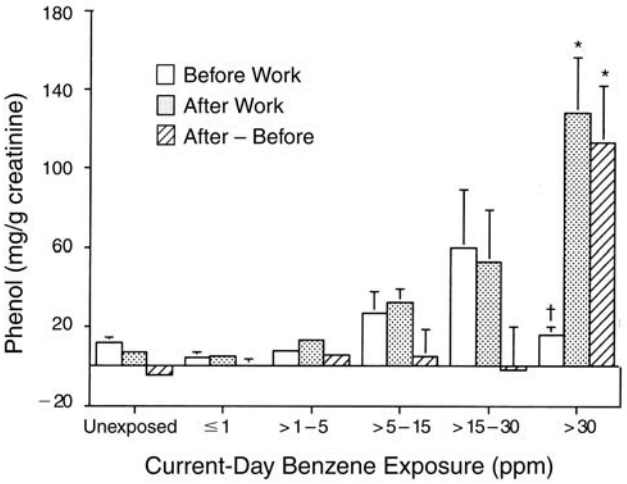

Figure 16. Urinary phenol levels (means ± SE) among all unexposed subjects (*n* = 51) and exposed subjects (*n* = 130) grouped according to the current-day benzene exposure. **P* ≤ 0.001, test for exposure-response trend based on an ANOVA linear contrast. †*P* ≤ 0.01, test for exposure-response trend based on an ANOVA linear contrast.

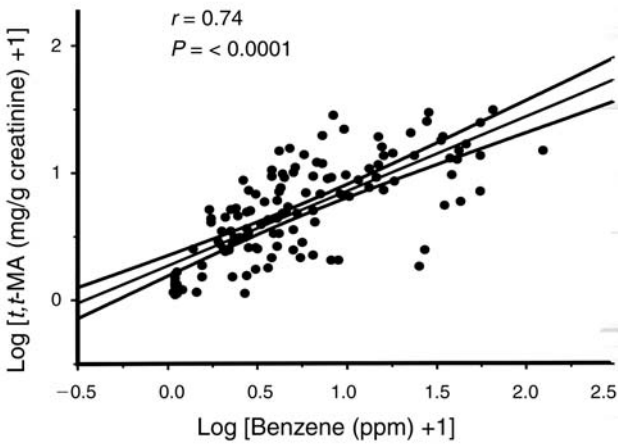

Figure 18. Relation between current-day benzene exposure and level of *t,t*-MA measured in urine samples collected from all exposed subjects (*n* = 130) after work. The middle line represents the average regression line and the 2 outer lines represent the 95% confidence interval (on a group basis).

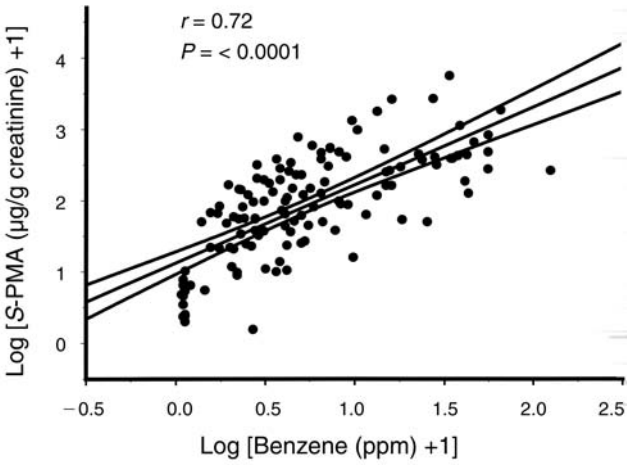

Figure 17. Relation between current-day benzene exposure and level of *S*-PMA measured in urine samples collected from all exposed subjects (*n* = 130) after work. The middle line represents the average regression line and the 2 outer lines represent the 95% confidence interval (on a group basis).

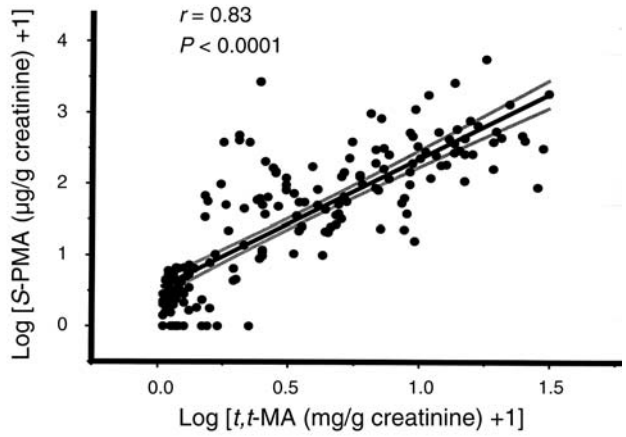

Figure 19. Relation between *S*-PMA and *t,t*-MA levels measured in urine samples collected from both unexposed and exposed subjects (*n* = 181) after work. The middle line represents the average regression line and the 2 outer lines represent the 95% confidence interval (on a group basis).

A significant correlation between urinary *t,t*-MA and *S*-PMA was also found in the combined group of 130 exposed subjects and 51 unexposed subjects ($r = 0.83$, $P < 0.0001$; Figure 19), similar to the strong correlation observed in the 25 exposed subjects in phase 1 of the study, in which benzene exposures were higher. Spearman rank order correlations were uniformly similar in magnitude to the parametric correlations for *t,t*-MA with *S*-PMA ($r = 0.85$, data not shown). However, their correlations with phenol were not that high: 0.41 for *t,t*-MA with phenol and 0.48 for *S*-PMA with phenol (data not shown).

Multiple regression analyses assessed exposure-response relations between the current-day benzene exposure level and the urinary metabolite biomarkers, with adjustment for several possible confounders: sex, age, cotinine level, and toluene exposure (Table 15). There was particular concern that toluene exposure might confound the benzene effect. However, a comparison of the results with and without toluene showed that toluene's impact was small and that the predominant effect was due to benzene. There was no evidence that smoking or age were confounders for the urinary metabolite variables (Table 15).

Table 15. Least-Squares Regression Analyses of Urinary Metabolite Levels by Current-Day Benzene Exposure for All Subjects (n = 181) With and Without Adjustment for Sex, Age, Cotinine Level, and Toluene Exposure[a]

Urinary Metabolite[b]	Benzene Unadjusted	Benzene Adjusted for Sex, Age, and Cotinine	Benzene Adjusted for Sex, Age, Cotinine, and Toluene
S-PMA			
Before work	0.058 ± 0.010[c]	0.058 ± 0.010[c]	0.047 ± 0.009[c]
After work	0.095 ± 0.010[c]	0.096 ± 0.010[c]	0.081 ± 0.008[c]
After − before	0.037 ± 0.006[c]	0.037 ± 0.006[c]	0.034 ± 0.006[c]
t,t-MA			
Before work	0.027 ± 0.003[c]	0.027 ± 0.003[c]	0.026 ± 0.003[c]
After work	0.046 ± 0.004[c]	0.046 ± 0.004[c]	0.041 ± 0.004[c]
After − before	0.019 ± 0.004[c]	0.019 ± 0.004[c]	0.016 ± 0.003[c]
Phenol			
Before work	0.021 ± 0.008[d]	0.021 ± 0.008[d]	0.023 ± 0.008[d]
After work	0.067 ± 0.007[c]	0.067 ± 0.007[c]	0.067 ± 0.007[c]
After − before	2.35 ± 0.245[c]	2.34 ± 0.247[c]	2.35 ± 0.251[c]
Ratio of *t,t*-MA to *S*-PMA	−0.003 ± 0.0009[e]	−0.003 ± 0.0009[e]	−0.002 ± 0.0009[f]

[a] The unexposed subjects were included in the analysis and were randomly assigned values between 0 and 0.01 ppm, which was the approximate lowest detection limit. Values are expressed as the regression coefficient ± SE.

[b] All metabolites were measured in mg/g creatinine except for *S*-PMA in μg/g. All data were log-transformed except for phenol, after work − before work.

[c] $P \leq 0.0001$, test for exposure-response trend.

[d] $P \leq 0.01$, test for exposure-response trend.

[e] $P \leq 0.001$, test for exposure-response trend.

[f] $P \leq 0.05$, test for exposure-response trend.

When the results for benzene exposure levels of 1 ppm or less were examined (Table 16), both *t,t*-MA and *S*-PMA showed significant exposure-response trends. Furthermore, an examination of the subgroup with benzene exposure of 0.5 ppm or lower showed a statistically significant increase in comparison with the unexposed group for the measurement of *S*-PMA after work. A few subjects with low benzene exposure, in the > 0 to 0.5 ppm group, had relatively high after-work concentrations of *S*-PMA (up to 44 μg/g creatinine) or *t,t*-MA (up to 1.1 mg/g creatinine) in the urine (data not shown) compared with the corresponding mean after-work levels of 1.87 μg/g creatinine and 0.26 mg/g creatinine, respectively, in unexposed subjects.

Further analysis was performed to determine whether the associations between benzene exposure and various biomarkers were linear or curvilinear. This was done by fitting models that included both linear and exposure-squared terms for benzene exposure. At the outset it should be noted that the scale of the biomarker variable may play a role in determining the shape of the exposure-response curve such that an alternative transformation of the biomarker variable could change the shape of the curve. We therefore tested the direction and significance of the exposure-squared component of the linear-quadratic curves for the urinary biomarkers using 3 metrics: arithmetic exposure and logarithmic biomarker, logarithmic exposure and logarithmic biomarker, and arithmetic exposure and arithmetic biomarker.

As it turned out, most of the *t,t*-MA and *S*-PMA biomarkers that showed negative exposure-squared terms, which imply convex curvilinearity (ie, a proportionally greater difference at low exposures than at high exposures), showed them with all 3 metrics used (Appendix B, Table B.6). Thus the sensitivity of *t,t*-MA and *S*-PMA as biomarkers of low-level benzene exposures (or the partial saturation of biomarker effects at high benzene exposures) is shown by the convex curvature of their responses.

For phenol, the after-work and after-work minus before-work measures were less sharply curvilinear. Thus phenol data showed less of the convex curvature that indicates low-exposure sensitivity than the other urinary biomarkers did. This same feature appeared in the exposure-response trend (Figure 16), in which there was not a clear increase in phenol levels for exposures below 5 ppm.

Table 16. Urinary Biomarkers for Unexposed and Low-Exposure Subjects, Exposure-Response Trend, and Comparison of Unexposed Subjects Versus Subjects Exposed to > 0 to 0.5 ppm Benzene[a]

Variable[b]	Exposure Level (ppm)			*P* for Exposure-Response Trend[c]	*P* for Unexposed vs >0 to 0.5 ppm[d]
	Unexposed	> 0 to 0.5	> 0.5 to 1		
Number of subjects	51	17	8		
Female (%)	53	94	38		
Smoker (%)[e]	31	6	25		
Current-day benzene exposure (ppm)	0	0.14±0.11	0.76±0.17		
S-PMA					
Before work	1.8±4.3	7.3±22	83.0±88.2	<0.0001	—
After work	1.87±1.86	6.49±11.3	60.8±48.4	<0.0001	0.001
After − before	0.07±5.4	−0.81±10.9	−22.2±52.9	—	—
t,t-MA					
Before work	0.34±0.58	0.26±0.54	1.36±0.65	<0.0001	—
After work	0.26±0.27	0.36±0.34	2.25±1.24	<0.0001	—
After − before	−0.08±0.62	0.10±0.51	0.89±0.87	0.006	—
Phenol					
Before work	11.7±21	1.22±2.81	11.5±24	—	—
After work	6.86±10	3.25±4.8	8.44±10.5	—	—
After − before	−4.85±21	2.02±5.8	−3.09±27	—	—
Cotinine level (µg/L)	560±1124	64±180	222±396	—	0.01

[a] Unless otherwise noted, values are presented as means ± SD.

[b] Unless otherwise noted, all metabolites were measured in mg/g creatinine except for *S*-PMA in µg/g.

[c] Least-squares regression analysis controlling for sex, age, cotinine level, and toluene exposure.

[d] Student *t* tests.

[e] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

Biotransformation Efficiencies of Benzene to Urinary S-PMA and t,t-MA When the exposed subjects were grouped according to 5 different levels of current-day benzene exposure, the metabolic efficiencies of benzene transformation to urinary *S*-PMA and *t,t*-MA decreased with increasing benzene exposure (Figure 20). Figures 21 and 22 show the interindividual variation in transformation of each 1 ppm benzene exposure to urinary *S*-PMA and *t,t*-MA, respectively, in exposed subjects grouped according to 3 different levels of current-day benzene exposure. The x-axes show the excretion of metabolites per benzene exposure in increments of 1 ppm, and the y-axes show the number of subjects with the corresponding rate of transformation. The *S*-PMA concentration of 12 µg/g creatinine per 1 ppm benzene exposure was frequent in all 3 subgroups of Figure 21. However, Figure 22 shows that transformation of 1 ppm benzene to a *t,t*-MA value of 1.3 mg/g creatinine was frequent in individuals in the subgroup with relatively low exposure (0.065 to 5 ppm benzene), and this *t,t*-MA value decreased to 0.4 mg/g creatinine in the high-exposure subgroup (> 15 to 122 ppm).

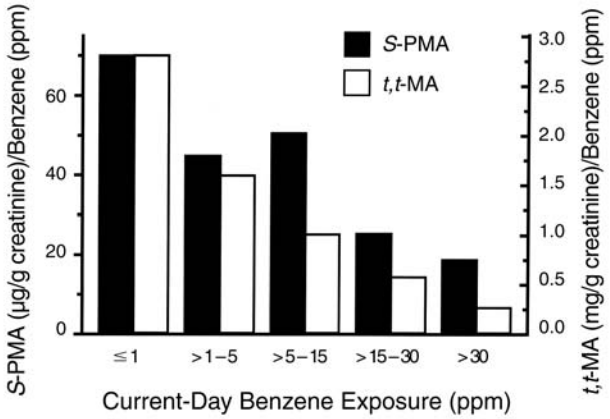

Figure 20. Excretion of urinary metabolites *S*-PMA and *t,t*-MA per 1 ppm benzene in all exposed subjects (n = 130) grouped according to current-day benzene exposure levels.

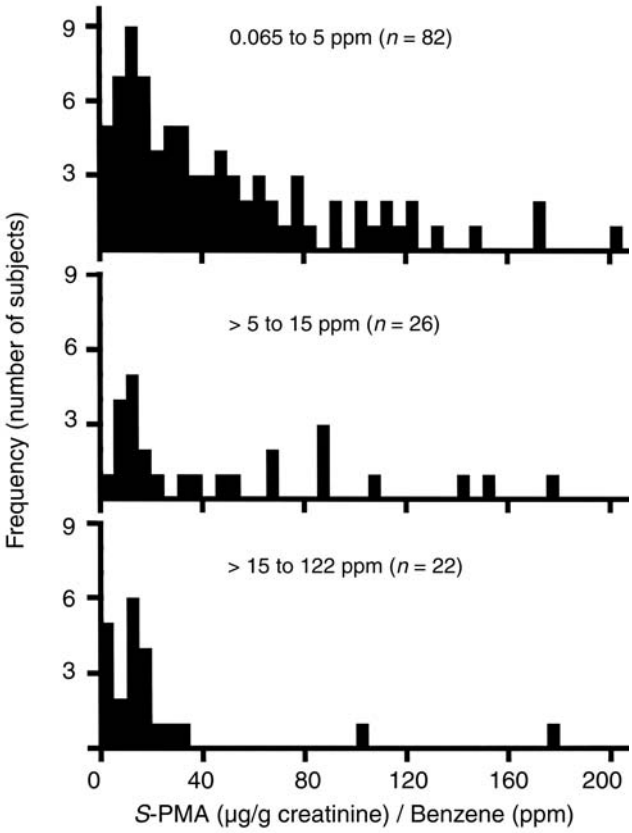

Figure 21. Interindividual variation in metabolic efficiency of benzene transformation into *S*-PMA in all exposed subjects (*n* = 130) grouped according to current-day benzene exposure levels.

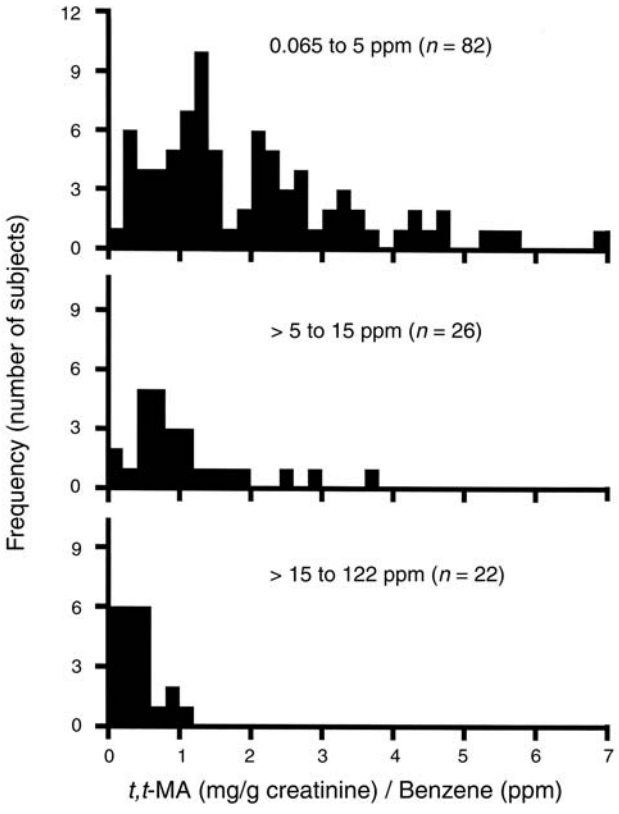

Figure 22. Interindividual variation in metabolic efficiency of benzene transformation into *t,t*-MA in all exposed subjects (*n* = 130) grouped according to current-day benzene exposure level.

VALIDATION OF ALBUMIN ADDUCTS OF BO AND 1,4-BQ

Reliability of Albumin Adduct Analyses

Ten quality control samples were prepared in phase 1 and in phase 2 of the study, and CVs were determined for the BO–Alb and 1,4-BQ–Alb adducts (Table 17). The pooled samples prepared for aliquots were different in phases 1 and 2 of the study; therefore, the 2 sets of data could not be combined for analysis and are presented here separately. The CVs ranged from 12.7% to 20.9% for 1,4-BQ–Alb and from 22.7% to 28.8% for BO–Alb. As previously discussed for urinary metabolites, these values represent not only the reliability and variability within a laboratory run, as is commonly assessed, but also the variability across runs in the laboratory, so they reflect day-to-day variability as well.

Furthermore, because multiple blood specimens were obtained from some subjects and multiple assays and

Table 17. Replicabilities of Albumin Adducts Measured in Pooled Quality Control Samples[a]

	Phase 1		Phase 2	
Adduct[b]	Mean ± SD	CV (%)	Mean ± SD	CV (%)
BO–Alb	0.146±0.033	22.7	0.191±0.055	28.8
1,4-BQ–Alb	1.234±0.156	12.7	0.750±0.157	20.9

[a] Based on measurements of 10 pooled samples performed at random in various runs.

[b] Both albumin adducts were measured in nmol/g albumin.

GC-MS injections were performed during analysis of adducts, ANOVA was performed to partition the total variability according to the different sources; that is, GC-MS injections were nested within assays, assays within blood specimens, and specimens within subjects. The results (Table 18) indicated that most of the variability in adduct levels was associated with differences in adduct levels among individual subjects. That is, 86.2% of the variance in BO–Alb and 94.0% of that in 1,4-BQ–Alb were associated with differences among subjects, while variation within subjects (from blood specimens obtained at different times in the 3-Monday study) accounted for 8.2% for BO–Alb and 4.2% for 1,4-BQ–Alb. In contrast, the precision of the assays (random errors in the assay plus injection) accounted for only 5.5% of the variation for BO–Alb and 1.8% of that for 1,4-BQ–Alb.

This partitioning of variability in adduct levels allows intraindividual and interindividual variability to be presented as the corresponding CVs (Table 18). We observed that the CV representing intraindividual variability was small, either 23% (for BO–Alb) or 18% (for 1,4-BQ–Alb), compared with the CV for differences between subjects, either 86% (for BO–Alb) or 99% (for 1,4-BQ–Alb). Finally, the assay-related CVs (assay plus injection) were 11% for 1,4-BQ–Alb and 19% for BO–Alb, indicating quite good precision.

Phase 1: Time Course Study

Blood samples were collected repeatedly from 11 subjects starting from 0 hours up to only 64 hours after exposure because the participating workers refused to donate blood for any longer period of time. Because this time frame is short compared with the normal turnover of serum albumin in humans (half-life of about 21 days), we did not anticipate that albumin adduct levels would have diminished significantly 64 hours after exposure. To investigate this effect, the correlation of adduct levels with time was investigated. Spearman coefficients were small for both types of adducts (0.0246 for BO–Alb adducts, $P = 0.916$; and -0.1454 for 1,4-BQ–Alb adducts, $P = 0.529$), indicating relatively little decay of albumin adducts during periods up to 64 hours after exposure.

Phase 1: Interindividual and Intraindividual Variability

The intraindividual variability over time with regard to the BO–Alb and 1,4-BQ–Alb adducts was analyzed in samples from 11 subjects gathered on 3 consecutive Mondays. The analysis of measurement consistency within individuals was indexed by CV. These analyses were unconventional in that they had to be based on a mixed model analysis of covariance, rather than the usual simple standard deviation, because of the fluctuation in benzene exposure levels for individuals from one Monday to the next. In effect, these analyses asked whether there were consistencies in albumin adduct levels within individuals over time when benzene exposure levels were held constant. The results of this analysis revealed that the geometric means of intraindividual CVs after adjustment for variations in benzene levels across the 3 weeks were relatively small: 10% for 1,4-BQ–Alb adducts and 20% for BO–Alb adducts (data not shown).

Table 18. Variance Components and Associated CVs for Different Sources of Variability in Adduct Levels[a]

Adduct	Source of Variability[b]	*df*[c]	Variance Component	% of Total	CV
BO–Alb	Subject	185	0.5503	86.2	0.857
	Specimen	40	0.0525	8.2	0.232
	Assay	85	0.0121	1.9	0.110
	Injection	142	0.0232	3.6	0.153
	Total	452	0.6381	100	0.945
1,4-BQ–Alb	Subject	185	0.6839	94.0	0.991
	Specimen	40	0.0304	4.2	0.176
	Assay	85	0.0036	0.5	0.060
	Injection	142	0.0094	1.3	0.097
	Total	452	0.7273	100	1.034

[a] Variance components were obtained as ANOVA estimates of the log-transformed adduct levels.

[b] *Subject* refers to variation between subjects, *specimen* to variation between blood specimens (within subjects), *assay* to variation between assays (within specimens), and *injection* to variation between GC-MS injections (within assays).

[c] Degrees of freedom.

Phase 2: Examination of Exposure-Response Relation

When the exposed subjects and unexposed subjects were compared with regard to the associations of BO–Alb and 1,4-BQ–Alb adducts with 4-week mean benzene exposure levels, the exposed subjects had significantly higher levels of both adducts ($P < 0.001$, Table 19). The albumin adduct data were broken down into subgroups based on the subjects' 4-week mean exposure levels (Table 20). A clear-cut exposure-response trend was observed ($P < 0.001$) across the broad range of benzene exposures; however, no statistically significant difference was detected between the unexposed group and the lowest exposed subgroup (0 to 5 ppm). These findings suggest that 1,4-BQ–Alb and BO–Alb adducts may not be sensitive biomarkers for benzene exposures below 5 ppm.

Table 19. Albumin Adduct Levels in All Unexposed and Benzene-Exposed Subjects

Variable	Unexposed Subjects (n = 51)	Exposed Subjects (n = 130)
Female (%)	53	52
Smoker (%)[a]	31	38
Age (years)	33.3±7.4	36.3±7.6
BO–Alb[b]	0.18±0.08	0.57±0.75[c]
1,4-BQ–Alb[b]	1.14±0.51	2.96±2.14[c]

[a] Includes self-reported smokers and subjects with cotinine levels > 100 µg/g creatinine.

[b] Both albumin adducts were measured in nmol/g albumin and reported as means ± SD for raw variables, but t tests were performed on the log-transformed data.

[c] $P \leq 0.001$ compared with unexposed subjects.

Scatter plots of the log-transformed levels of BO–Alb and 1,4-BQ–Alb adducts versus the log-transformed exposure levels of individual subjects (Figure 23) show that logarithmic transformation normalized the variance over the full range of exposures, as required for regression analyses. The residuals were unremarkable with 2 possible exceptions

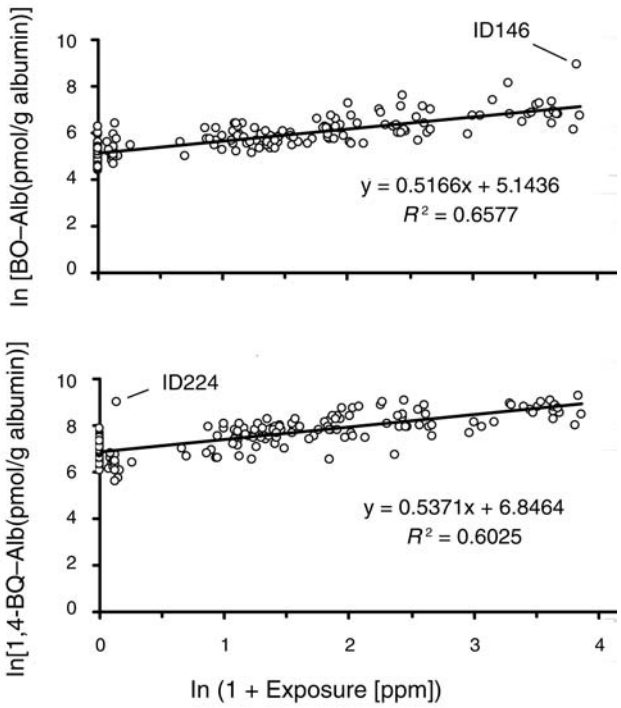

Figure 23. Adduct-exposure relation of BO-Alb adducts (top) and 1,4-BQ–Alb adducts (bottom) with 4-week mean benzene exposure after (natural) logarithmic transformation of both variables for all subjects (n = 181). The independent variable is the logarithm of 1 plus the subject-specific geometric mean. See text for discussion of outliers ID 146 (top) and ID 224 (bottom).

Table 20. Albumin Adduct Levels in Subjects Grouped According to 4-Week Mean Benzene Exposure[a]

Variable	Unexposed	4-Week Mean Benzene Exposure (ppm) >0 to 5	>5 to 15	>15 to 30	>30
Number of subjects	51	73	33	8	16
Female (%)	53	55	33	88	63
Smoker (%)[b]	31	36	55	0	38
Age (years)	33	37	36	39	33
4-Week mean benzene exposure (ppm)	0.004±0.003	2.26±1.35	8.67±2.44	19.9±3.1	51.8±43.3
BO–Alb[c]	0.18±0.08	0.31±0.13	0.60±0.38	0.87±0.46	1.50±1.73
1,4-BQ–Alb[c]	1.1±0.5	1.8±1.2	3.7±1.9	3.3±1.7	6.3±1.9

[a] Unless otherwise noted, values are presented as means ± SD.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

[c] Both albumin adducts were measured in nmol/g albumin. $P \leq 0.001$, test for exposure-response trend across all subgroups.

(IDs 146 and 224). Again, for both adducts clear trends of increasing levels are seen with increasing benzene exposure. The plots also indicate large background levels of both adducts in the unexposed subjects, as observed previously (Yeowell-O'Connell et al 1998).

Results of the regression analyses for BO–Alb revealed significant associations with exposure as well as age, sex, and the interaction terms, exposure × age and sex × age (Table 21). The adjusted R^2 = 0.6845 indicates that the model explained more than two thirds of the variability of BO–Alb. However, when all covariates and interaction terms were removed (leaving only exposure as an independent variable), the adjusted model correlation was only marginally affected, R^2 = 0.6558, indicating that age and sex only explained about 3% of the variability of BO–Alb. Furthermore, residual analysis revealed 1 extremely influential exposed subject (ID 146, a 22-year-old smoking male) identified in Figure 23 (studentized residual = 3.254, Cook distance = 0.215). When the data for this worker were removed from the analysis, the final model was quite different (Table 21); in this analysis only exposure, age, and exposure × age were retained and the adjusted model R^2 = 0.6702.

Table 21. Final Least-Squares Regression Models for ln(BO–Alb Adducts)

Variable[a]	Parameter Estimate ± SE	*P* Value
All Subjects Included (Adjusted R^2 = 0.6845)		
Intercept	4.3570±0.3640	0.0001
Exposure [ln(1+GM exposure)][b]	0.9301±0.1205	0.0001
Age	0.0219±0.0099	0.0286
Sex	0.6206±0.3471	0.0755
Exposure×age	−0.0122±0.0035	0.0006
Sex×age	−0.0163±0.0094	0.0846
After 1 Influential Exposed Subject [ID 146] Was Removed (Adjusted R^2 = 0.6702)		
Intercept	4.9600±0.2233	0.0001
Exposure [ln(1+GM exposure)][b]	0.8065±0.1215	0.0001
Age	0.0060±0.0063	0.3488
Exposure×age	−0.0090±0.0035	0.0104
Unexposed Subjects Only (Adjusted R^2 = 0.0554)		
Intercept	3.7835±0.5635	0.0001
Age	−0.0362±0.01502	0.0199
Sex	1.3033±0.6396	0.0472
Sex×age	−0.0359±0.0181	0.0532

[a] All subjects, *n* = 181; unexposed subjects, *n* = 51.

[b] *GM* refers to the subject-specific 4-week geometric mean exposure to benzene in ppm.

Because the interaction of exposure and age complicated interpretation of the adduct-exposure relation, the effects of exposure and age on BO–Alb were further investigated by regressing adduct level on exposure after first classifying workers into the youngest, medium, and oldest tertiles of age. The results revealed an apparent trend in that the slope of the linear relation between ln(BO–Alb) and ln(1 + Geometric Mean of Exposure) decreased from 0.56 for the youngest tertile to 0.43 for the oldest tertile (Table 22).

Regression analysis of 1,4-BQ–Alb showed significant associations with exposure, age, and sex; however, in this case no interaction terms were retained in the final model (Table 23). The levels of 1,4-BQ were significantly higher in male workers and lower in older workers. The adjusted model R^2 value of 0.6637 was quite similar to that for BO–Alb (R^2 = 0.6845). Also, when all covariates and interaction terms were removed (leaving only exposure as the independent variable), the adjusted model R^2 equaled 0.6004. This indicates that age and sex explained only about 6% of the variability of BQ–Alb. Removal of a possible outlier identified in Figure 23 (ID 224, studentized residual = 4.887, Cook distance = 0.090) did not lead to a final model materially different from that shown in Table 23 (results not shown). Further analyses stratified by age and sex were unremarkable (results not shown).

Table 22. Stratified Analysis of ln(BO–Alb Adducts) Regressed on Exposure by Age Tertile for All Subjects[a]

Age Tertile	Mean Age (years)	Age Range (years)	Intercept ± SE[b]	Slope for Exposure ± SE[b]
Lowest (*n* = 59)	25.9	18–33	5.1226±0.0680	0.5581±0.0373
Medium (*n* = 56)	36.4	34–39	5.1964±0.0846	0.5130±0.0598
Highest (*n* = 65)	42.9	40–52	5.1736±0.0791	0.4260±0.0446

[a] After 1 influential exposed subject (ID 146) was removed.

[b] Least-squares regression estimates.

Table 23. Final Least-Squares Regression Models for ln(1,4-BQ–Alb Adducts)

Variable[a]	Parameter Estimate±SE	*P* Value
All Subjects Included (Adjusted R^2 = 0.6637)		
Intercept	4.1731±0.1770	0.0001
Exposure [ln(1+GM exposure)][b]	0.5278±0.0298	0.0001
Age	−0.0132±0.0045	0.0039
Sex	0.3094±0.0716	0.0001
Unexposed Subjects Only (Adjusted R^2 = 0.2005)		
Intercept	6.7367±0.0790	0.0001
Sex	0.4236±0.1151	0.0006

[a] All subjects, n = 181; unexposed subjects, n = 51.

[b] *GM* refers to the subject-specific 4-week geometric mean exposure to benzene in ppm.

Because of the strong effect of benzene exposure on albumin adducts, we repeated the regression analyses of all covariates among only the 51 unexposed subjects. The results again pointed to significant but weak effects of age, sex, and their interaction on levels of BO–Alb (Table 21) and to a rather strong effect of sex on levels of 1,4-BQ–Alb (Table 23).

In addition to the 4-week mean benzene exposure levels, lifetime cumulative benzene exposure levels were used to examine the exposure-response relations (Table 24) and revealed strong associations with both BO–Alb and 1,4-BQ–Alb adducts. Even in the group with the lowest lifetime cumulative benzene exposure (mean, 16 ppm-years), both adduct levels were significantly higher than those in the unexposed subjects. Further regression analyses with adjustment for sex, age, cotinine level, and toluene exposure still resulted in strong associations between albumin adducts and lifetime cumulative benzene exposure ($P < 0.001$, data not shown).

Further analysis was conducted to determine the relative contributions of benzene exposure duration (Table 25) and exposure intensity measured as mean exposure level per year (Table 26) to the observed associations between levels of lifetime cumulative benzene exposure and albumin adducts. Neither BO–Alb nor 1,4-BQ–Alb increased consistently with duration of exposure; however, both adducts showed strong associations with exposure intensity. This was confirmed by regression analyses adjusting for sex, age, cotinine level, and toluene exposure, in which both types of adducts had a strong exposure-response association with exposure intensity ($P < 0.0001$) but not with exposure duration (data not shown).

Table 24. Albumin Adduct Levels in Subjects Grouped According to Lifetime Cumulative Benzene Exposure[a]

		Lifetime Cumulative Exposure (ppm-years)			
Variable	Unexposed	> 0 to < 30	30 to < 50	50 to < 100	≥100
Descriptive characteristics					
Number of subjects	51	30	35	31	34
Female (%)	53	30	74	58	44
Smoker (%)[b]	31	53	29	36	38
Age (years)	33.3±7.4	33.1±9.5	37.7±3.2	38.7±4.8	35.3±9.9
Benzene exposure					
Lifetime cumulative (ppm-years)	0	16.0±8.0	40.8±6.0	73.9±14.4	187±117
4-Week mean (ppm)	0.004±0.003	3.82±2.8	2.67±2.7	8.85±11.0	28.1±36.2
Adduct levels					
BO–Alb[c]	0.18±0.08	0.36±0.17	0.30±0.14	0.52±0.37	1.06±1.28
1,4-BQ–Alb[c]	1.1±0.5	2.4±1.2	1.7±1.1	2.9±2.1	4.8±2.4

[a] Unless otherwise noted, values are presented as means ± SE. Both albumin adducts were measured in nmol/g albumin.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

[c] $P < 0.001$, test for exposure-response trend based on an ANOVA linear contrast.

Table 25. Albumin Adduct Levels in Subjects Grouped According to Benzene Exposure Duration[a]

Variable	Unexposed	Exposure Duration (years)			
		<5	5 to <10	10 to <18	≥18
Descriptive characteristics					
Number of subjects	51	44	38	28	20
Female (%)	53	39	50	68	65
Smoker (%)[b]	31	45	45	25	30
Age (years)	33.3±7.4	32.2±9.5	38.8±5.8	36.5±4.3	40.0±5.6
Benzene exposure					
Lifetime cumulative (ppm-years)	0	78±86	84±82	77±122	90±55
Intensity (ppm-years)	0	28.2±40.2	13.3±13.0	6.4±10.5	4.6±2.9
4-Week mean (ppm)	0.004±0.003	14.5±16.7	16.2±34.6	3.1±4.0	5.0±5.9
Adduct levels					
BO–Alb	0.18±0.08	0.82±1.17	0.53±0.37	0.35±0.26	0.40±0.28
1,4-BQ–Alb	1.1±0.5	3.9±2.4	3.2±1.7	1.9±1.7	2.0±1.7

[a] Unless otherwise noted, values are presented as means ± SE. Both albumin adducts were measured in nmol/g albumin.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

Table 26. Albumin Adduct Levels in Subjects Grouped According to Benzene Exposure Intensity[a]

Variable	Unexposed	Exposure Intensity (ppm-years)			
		>0 to <5	5 to <15	15 to <40	≥40
Descriptive characteristics					
Number of subjects	51	54	36	29	11
Female (%)	53	54	53	48	55
Smoker (%)[b]	31	35	42	45	27
Age (years)	33.3±7.4	35.2±7.6	39.3±5.2	36.3±8.7	31.4±8.6
Benzene exposure					
Lifetime cumulative (ppm-years)	0	32±21	74±51	123±65	237±188
Mean years of exposure	0	11.2±6.4	9.8±6.0	4.4±1.9	4.0±3.2
4-Week mean (ppm)	0.004±0.003	3.07±2.9	5.89±4.8	17.4±15.5	50.6±55.4
Adduct levels					
BO–Alb[c]	0.18±0.08	0.32±0.16	0.45±0.26	0.94±1.33	1.17±0.80
1,4-BQ–Alb[c]	1.1±0.5	2.0±1.2	2.5±1.7	4.1±2.4	6.0±2.0

[a] Unless otherwise noted, values are presented as means ± SE. Both albumin adducts were measured in nmol/g albumin.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

[c] $P < 0.001$, test for exposure-response trend based on an ANOVA linear contrast.

In addition to their correlation with benzene exposure levels, BO–Alb and 1,4-BQ–Alb levels were highly correlated with each other (Figure 24).

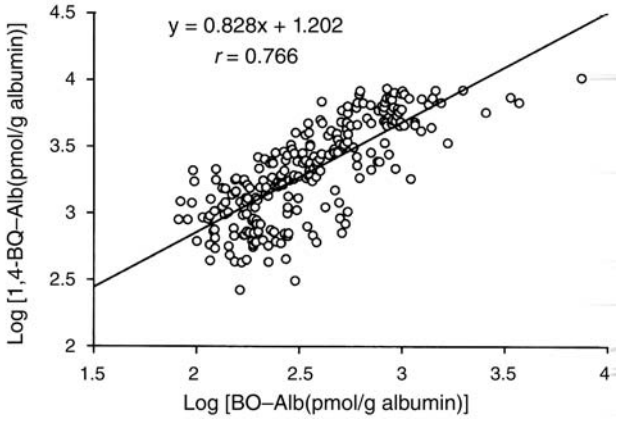

Figure 24. Correlation between log-transformed levels of BO–Alb adducts and 1,4-BQ–Alb adducts for all subjects (n = 181).

VALIDATION OF CHROMOSOMAL ABERRATION ASSAYS

Phase 1: FISH Assay

The results of various FISH analyses performed on blood cells obtained from the highly exposed benzene workers recruited during phase 1 are described briefly in this section by cell type. (Appendix B gives the full results based on current 5-week mean exposure [Table B.7] and on lifetime cumulative exposure [Table B.8].)

Unstimulated G_0 Lymphocytes and Granulocytes Using the tandem probe assay for chromosome 1 and the single probe assay for chromosome 7, the frequencies of breakage affecting the 1cen-1q12 region on chromosome 1 and aneuploidy affecting chromosomes 1 and 7 were determined in interphase cells. No increase in hyperdiploidy for chromosomes 1 and 7 or breakage affecting the 1cen-1q12 region was detected in the granulocytes obtained from the benzene-exposed subjects as compared with the unexposed subjects (Tables B.7 and B.8). Nor was an increase in hypodiploidy observed. In the unstimulated G_0 lymphocytes, marginal exposure-related increases in hyperdiploidy were seen with both the chromosome 1 and chromosome 7 probes according to 5-week mean benzene exposure levels (Table B.7). These increases for both probes achieved statistical significance when analyzed according to cumulative benzene exposure levels (P = 0.03, Table B.8). No increase in breakage in the 1cen-1q12 region or hypodiploidy was observed in these cells. For both the G_0 lymphocyte and granulocyte controls irradiated in vitro with 0 or 2 Gy of ionizing radiation, consistent increases in breakage were seen in both cell types as compared with unirradiated controls (Table B.9).

48-Hour Lymphocyte Cultures The tandem probes for chromosome 1 were also used to determine the frequency of hyperdiploidy and the breakage affecting the 1cen-1q12 region in the 48-hour cultured interphase lymphocytes. No significant increases in hyperdiploidy and breakage in this heterochromatic region were seen in the lymphocytes from the exposed subjects (Tables B.7 and B.8). In fact, for both hyperdiploidy and 1cen-1q12 breakage, frequencies in the unexposed subjects appeared to be somewhat higher than those in the exposed subjects. Furthermore, no increase in hypodiploidy for chromosome 1 was seen. Positive and negative control lymphocytes irradiated with 0 or 2 Gy of ionizing radiation or lymphocytes treated in culture with the aneuploidy-inducing agent diethylstilbestrol (at 0 or 10 μM) exhibited strong and consistent increases in both breakage (radiation) and hyperdiploidy (diethylstilbestrol and to a lesser extent radiation).

72-Hour Lymphocyte Cultures A single chromosome probe was used to detect gain or loss of chromosome 7 in the 72-hour lymphocyte cultures. No increases in either hyperdiploidy or hypodiploidy for this chromosome were seen in the exposed subjects as compared with the unexposed controls (Tables B.7 and B.8). Again, the expected results were seen in the negative and positive control slides—cultured lymphocytes treated with 0 and 10 μM diethylstilbestrol, respectively. Highly significant increases in hyperdiploidy and a weak increase of approximately 2-fold in hypodiploidy were seen in the diethylstilbestrol-treated positive control cells (Table B.9).

Phase 2: Examination of Exposure-Response Relation

The relation between benzene exposure and chromosomal aberrations was analyzed both for 4-week mean benzene concentrations and for lifetime cumulative exposures. Chromosomal aberration data were combined from samples collected during phases 1 and 2 of the study and analyzed mainly according to 3 categories: (1) total chromatid-type aberrations (except gaps), which include chromatid breaks, deletions, and exchanges; (2) total chromosomal-type aberrations (except gaps), including breaks, acentric fragments, minute fragments, rings, and dicentric fragments; and (3) total aberrations containing all chromatid-type and chromosomal-type aberrations except gaps. Individual categories of aberrations that occurred at substantial frequencies have been reported. It should be noted, however,

that gaps are not usually considered a reliable indicator of cytogenetic damage. The results are given below.

Chromosomal Aberrations In the results analyzed using 4-week mean exposures, significant increases were observed for chromatid breaks, total chromatid-type aberrations, total chromosomal-type aberrations, and total aberrations (Table 27). These increases were due primarily to an increase in chromatid breaks, although other aberration categories such as chromosomal breaks appeared to contribute to the trend. Unexpectedly, a modest increase in the mitotic index (metaphases per 1000 cells) was also seen with increasing benzene exposure. This was due almost entirely to an increase in the number of metaphases observed in the samples from workers with the highest exposure. Furthermore, when adjustments were made for possible confounding variables and benzene exposure was treated as a continuous variable, the results were somewhat different (Table 28). There were moderate associations of benzene exposure with chromatid gaps and chromosomal breaks, but not for any of the grouped categories of total chromatid aberrations, total chromosomal aberrations, or total aberrations.

When the low portion of the benzene exposure spectrum (< 0.5 ppm) was examined, there were positive associations for total chromatid aberrations, total chromosomal aberrations, total aberrations, chromatid breaks, and acentric fragments (Table 29). Analysis of a linear-quadratic exposure-response model did not show quadratic curvature for any category of chromosomal aberration (data not shown).

An analysis by lifetime cumulative benzene exposure (Table 30) showed moderate to strong associations for total chromatid aberrations, total chromosomal aberrations, total aberrations, chromatid breaks, and acentric fragments. The regressions of the chromosomal aberration data on lifetime cumulative benzene exposure (Appendix A, Table A.13) showed weak to moderate associations with total chromatid aberrations, total chromosomal aberrations, total aberrations, chromatid gaps, chromatid breaks, and chromosomal breaks. The associations with chromosomal aberrations were analyzed further by the 2 components of cumulative benzene exposure, exposure

Table 27. Chromosomal Aberration Outcomes and Aneuploidy Grouped by Subjects According to 4-Week Mean Benzene Exposures[a]

		4-Week Mean Exposure (ppm)			
Variable	Unexposed	> 0 to 5	> 5 to 15	>15 to 30	>30 ppm
Number of subjects	51	73	33	8	16
Female (%)	53	55	33	88	63
Smoker (%)[b]	31	36	55	0	38
Age (years)	33.3±7.4	36.6±6.4	36.3±9.6	38.5±8.5	33.4±7.7
4-Week mean benzene exposure	0.004±0.003	2.26±1.35	8.67±2.44	19.9±3.1	51.8±43.3
Total chromatid aberrations (except gaps)[c]	1.20±1.36	2.01±1.5	2.00±1.4	1.43±1.3	2.25±1.8
Total chromosomal aberrations (except gaps)[d]	0.59±1.3	0.97±1.2	0.74±1.0	0.71±1.3	1.44±1.3
Total aberrations (except gaps)[c]	1.78±2.1	2.99±2.1	2.74±2.0	2.14±2.1	3.69±2.5
Chromatid gaps	1.37±1.7	0.59±1.0	0.71±1.79	1.00±1.0	2.31±2.0
Chromatid breaks[c]	1.20±1.4	1.99±1.5	2.00±1.4	1.43±1.3	2.25±1.8
Chromosomal breaks	0.20±0.50	0.25±0.63	0.23±0.56	0.57±0.98	0.50±0.52
Hypodiploidy (45 chromosomes)	0.22±0.54	0.12±0.33	0.39±0.84	0.29±0.49	0.38±0.72
Hypodiploidy (≤44 chromosomes)	2.7±2.7	7.0±7.1	7.6±9.6	5.5±5.2	3.5±3.1
Hyperdiploidy (≥47 chromosomes)	0.06±0.24	0.16±0.50	0.13±0.34	0	0.06±0.25
Aneuploidy (45 or ≥47 chromosomes)	0.27±0.57	0.29±0.61	0.52±0.96	0.29±0.49	0.44±0.73
Total aneuploidy (≤45 and ≥47 chromosomes)	3.0±2.7	7.3±7.2	8.1±9.5	5.8±5.1	3.9±3.5
Metaphases/1000 cells[c]	45±26	49±25	47±20	45±22	72±27

[a] Unless otherwise noted, the values are reported as means (± SD); chromosomal aberrations and ploidy variables are number per 100 cells; the statistical tests were performed on square-root transformed data. Exposure-response trends were based on ANOVA linear contrast.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

[c] $P \leq 0.01$, test for exposure-response trend.

[d] $P \leq 0.05$, test for exposure-response trend.

Table 28. Regression Analyses of Chromosomal Aberrations and Aneuploidy by 4-Week Mean Benzene Exposure, With and Without Adjustment for Sex, Age, Cotinine Level, and Toluene Exposure[a]

Chromosomal Aberrations and Aneuploidy[b]	Unadjusted	Adjusted
Total chromatid aberrations (except gaps) ($\sqrt{}$)	0.004 ± 0.003	0.002 ± 0.003
Total chromosomal aberrations (except gaps ($\sqrt{}$)	0.004 ± 0.003	0.0004 ± 0.003
Total aberrations (except gaps) ($\sqrt{}$)	0.006 ± 0.004	0.003 ± 0.004
Chromatid gaps ($\sqrt{}$)	0.008 ± 0.003[c]	0.010 ± 0.003[d]
Chromatid breaks ($\sqrt{}$)	0.004 ± 0.003	0.002 ± 0.003
Chromosomal breaks ($\sqrt{}$)	0.004 ± 0.002[c]	0.004 ± 0.002[c]
Hypodiploidy (45 chromosomes) ($\sqrt{}$)	0.003 ± 0.002[c]	0.003 ± 0.002[c]
Hypodiploidy (≤44 chromosomes) ($\sqrt{}$)	0.001 ± 0.007	−0.003 ± 0.007
Hyperdiploidy (≥47 chromosomes) ($\sqrt{}$)	−0.0008 ± 0.001	−0.0009 ± 0.001
Aneuploidy (45 or ≥47 chromosomes) ($\sqrt{}$)	0.002 ± 0.002	0.002 ± 0.002
Total aneuploidy (≤45 and ≥47 chromosomes) ($\sqrt{}$)	0.002 ± 0.007	−0.002 ± 0.007
Stimulated cells/1000 cells[e]	0.94 ± 0.78	1.13 ± 0.80
Metaphases/1000 cells	0.50 ± 0.15[d]	0.50 ± 0.15[d]

[a] All 181 subjects were included in the analysis; the 51 unexposed subjects were randomly assigned values between 0 and 0.01 ppm, which was the approximate lowest detection limit. The log-transformed values for cotinine and toluene were used in the analysis. Values are reported as the least-squares regression coefficient ± SE.

[b] ($\sqrt{}$) = square root transformation using the square root of (x + 3/8), where x is the raw variable.

[c] $P \leq 0.05$, test for exposure-response trend.

[d] $P \leq 0.001$, test for exposure-response trend.

[e] Lymphocytes that have been stimulated to divide using phytohemagglutinin per 1000 cells evaluated.

duration (Table 31) and exposure intensity (Table 32). Regression analyses for these components (Appendix A, Table A.16) showed that exposure duration was predictive of total chromatid aberrations, total aberrations, chromatid breaks, and acentric fragments, while exposure intensity was predictive of chromatid gaps and chromosomal breaks. It is not clear whether the difference in attribution for the 2 sets of variables is meaningful or due to chance fluctuations.

As with the FISH analyses, positive and negative control slides consisting of cultures treated with 0 and 2 Gy of radiation were coded and scored along with the study slides. As expected, strong increases in structural aberrations and modest increases in aneuploid cells were seen in the irradiated cells as compared with the controls (Appendix B, Table B.10). The negative results observed in phase 1 differed substantially from positive results that had been obtained in earlier studies (Rupa et al 1995; Marcon et al 1999); therefore, a small follow-up study was performed to determine whether the tandem FISH approach could detect 1cen-1q12 breaks in workers exposed to lower levels of benzene. Coded slides from approximately 10 workers in each of 4 exposure categories were hybridized with the tandem FISH probes and evaluated for aberrations involving chromosome 1. The results are shown in Appendix Table B.11. Dose-related increases in both 1cen-1q12 breakage and hyperdiploidy involving chromosome 1 were seen.

Aneuploidy The aneuploidy data analyzed by 4-week mean benzene exposure levels revealed that only hypodiploidy (45 chromosomes) had a positive association with benzene exposure (see Tables 27 and 28). No changes were observed for any type of aneuploidy when unexposed subjects were compared with workers exposed to benzene at or below 0.5 ppm (see Table 29). However, analyses by lifetime cumulative benzene exposure (see Table 30) did show weak to strong associations for aneuploidy (45 and ≥ 47 chromosomes) and total aneuploidy (≤ 45 and ≥ 47 chromosomes). Regression analyses, with or without adjustment for sex, age, cotinine level, and toluene exposure, continued to show moderate associations (Appendix A, Table A.13). Further analyses showed that both hypodiploidy (45 chromosomes) and aneuploidy (45 or ≥ 47 chromosomes) were strongly associated with exposure intensity (mean benzene exposure level per year), but not with exposure duration (Appendix A, Table A.16).

Table 29. Chromosomal Aberrations and Aneuploidy in Unexposed Subjects and Subjects with 4-Week Mean Benzene Exposures of 0.5 ppm or Lower[a]

Variable	Unexposed	Exposed (> 0 to 0.5 ppm)	*P* Value[b]
Number of subjects	51	16	NA
Female (%)	53	100	NA
Smoker (%)[c]	31	0	NA
Age (years)	33.3 ± 7.4	36.2 ± 3.2	—
4-Week mean benzene exposure	0.004 ± 0.003	0.14 ± 0.04	—
Total chromatid aberrations (except gaps)	1.20 ± 1.36	2.19 ± 1.38	0.01
Total chromosomal aberrations (except gaps)	0.59 ± 1.25	1.44 ± 1.41	0.008
Total aberrations (except gaps)	1.78 ± 2.05	3.63 ± 2.09	0.001
Chromatid gaps	1.37 ± 1.66	0.63 ± 0.81	0.09
Chromatid breaks	1.20 ± 1.36	2.19 ± 1.38	0.01
Chromosomal breaks	0.20 ± 0.49	0.38 ± 0.5	—
Acentric fragments	0.39 ± 0.98	1.06 ± 1.29	0.01
Hypodiploidy (45 chromosomes)	0.22 ± 0.54	0.19 ± 0.40	—
Hypodiploidy (≤ 44 chromosomes)	2.71 ± 2.69	3.00 ± 4.24	—
Hyperdiploidy (≥ 47 chromosomes)	0.06 ± 0.24	0.06 ± 0.25	—
Aneuploidy (45 or ≥ 47 chromosomes)	0.27 ± 0.57	0.25 ± 0.45	—
Total aneuploidy (≤ 45 and ≥ 47 chromosomes)	2.98 ± 2.69	3.25 ± 4.5	—
Metaphases/1000 cells	45 ± 26	38 ± 21	—

[a] Unless otherwise noted, the values are reported as means (± SD); chromosomal aberrations and ploidy variables are number per 100 cells; the statistical tests were performed on square-root transformed data. The exposure-response trend was tested by an ANOVA linear contrast which did not control for other factors.

[b] Least-squares regression analysis controlling for sex, age, year, smoking, cotinine level, and toluene exposure. NA = not applicable. Dashes indicate $P > 0.10$.

[c] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

CORRELATIONS BETWEEN BIOMARKERS

Correlations Between Urinary Metabolites and Albumin Adducts

In addition to being significantly correlated with each other ($r = 0.74$), BO–Alb adducts and 1,4-BQ–Alb adducts were strongly correlated with the after-work levels of urinary *t,t*-MA and *S*-PMA, with Spearman coefficients between 0.69 and 0.75 (Table 33). These albumin adducts were also significantly correlated with the after-work levels of phenol, HQ, and CAT, with Spearman coefficients between 0.35 and 0.54.

Correlations Between Urinary Metabolites, Albumin Adducts, and Chromosomal Aberrations

The initial correlation analyses of the raw data showed either weak or no associations of urinary metabolites and albumin adducts with chromosomal aberrations (Table 34). However, during further analyses of benzene biomarkers in urine, we found that the ratios between metabolites and corresponding current-day benzene exposure levels varied extremely from subject to subject, suggesting that some workers were poor and some were efficient in benzene metabolism (Figures 21 and 22). The precursor of *t,t*-MA, *t,t*-muconaldehyde, is myelotoxic, and its production has been implicated in benzene-induced leukemia. Therefore, differences in efficiency of *t,t*-MA metabolism may put subjects at different risks of developing benzene toxicity. This may be also true for HQ metabolism because HQ is a precursor to one or more of the toxic metabolites of benzene formed via CYP2E1 activation.

For these reasons, 2 urinary metabolites, HQ and *t,t*-MA, were further examined as to whether they were predictive of chromosomal aberrations. Albumin adducts were also included because they are the only biomarkers in our study to reflect integrated exposures for longer periods.

Table 30. Chromosomal Aberrations and Aneuploidy Grouped by Subject According to Lifetime Cumulative Benzene Exposure[a]

Variable	Unexposed	Lifetime Cumulative Exposure (ppm-years)			
		> 0 to <30	30 to <50	50 to <100	≥100
Number of subjects	51	30	35	31	34
Female (%)	53	30	74	58	44
Smoker (%)[b]	31	53	29	36	38
Age (years)	33.3±7.4	33.1±9.5	37.7±3.2	38.7±4.8	35.3±9.9
Benzene exposure					
Lifetime cumulative (ppm-years)	0	16.0±8.0	40.8±6.0	73.9±14.4	187±117
4-Week mean (ppm)	0.004±0.003	3.82±2.8	2.67±2.7	8.85±11.0	28.12±36.2
Total chromatid aberrations (except gaps)[c]	1.20±1.36	1.55±1.30	1.80±1.35	2.50±1.61	2.18±1.70
Total chromosomal aberrations (except gaps)[d]	0.59±1.25	0.48±0.83	1.11±1.08	1.20±1.40	1.00±1.17
Total aberrations (except gaps)[e]	1.78±2.14	2.03±1.61	2.91±1.79	3.70±2.41	3.18±2.31
Chromatid gaps	1.4±1.7	0.5±1.1	0.5±0.7	0.7±1.4	1.7±2.1
Chromatid breaks[e]	1.1±1.3	1.5±1.3	1.8±1.3	2.5±1.6	2.2±1.6
Chromosomal breaks[f]	0.22± 0.50	0.03±0.19	0.34±0.64	0.43±0.63	0.42±0.79
Acentric fragments[f]	0.39± 0.98	0.38± 0.73	0.77±0.88	0.73±1.1	0.58±0.79
Hypodiploidy (45 chromosomes)	0.22± 0.54	0.07±0.26	0.14±0.36	0.23±0.63	0.45±0.79
Hypodiploidy (≤44 chromosomes)[d]	2.7±2.7	6.4±6.8	6.3±7.1	8.2±7.7	5.6±8.0
Hyperdiploidy (≥47 chromosomes)	0.06±0.24	0.10±0.31	0.11±0.40	0.23±0.63	0.09±0.29
Aneuploidy (45 or ≥47 chromosomes)[f]	0.27±0.57	0.17±0.38	0.26±0.61	0.47±0.82	0.55±0.90
Total aneuploidy (≤45 and ≥47 chromosomes)[e]	3.0± 2.7	6.6±6.8	6.6±7.4	8.7±7.5	6.1±8.0
Metaphases/1000 cells [f]	45± 26	47±24	48±25	55±30	54±22

[a] Unless otherwise noted, the values are reported as means (± SD); chromosomal aberrations and ploidy variables are number per 100 cells; the statistical tests were performed on square-root transformed data. The exposure-response trend was tested by an ANOVA linear contrast which did not control for other factors.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

[c] $P \leq 0.0001$, test for exposure-response trend.

[d] $P \leq 0.01$, test for exposure-response trend.

[e] $P \leq 0.001$, test for exposure-response trend.

[f] $P \leq 0.05$, test for exposure-response trend.

The responsiveness was defined either by the regression of current-day benzene exposure on the difference between before-work and after-work levels of *t,t*-MA and HQ, or by the regression of 4-week mean benzene exposure on albumin adducts. The residuals from the predicted amounts of HQ, *t,t*-MA, and albumin adducts were used as indicators of responsiveness, and the residual values were correlated with chromosomal aberration outcomes.

Both HQ and, to a lesser extent, *t,t*-MA residuals were positively associated with chromosomal aberration frequency (Table 35). A high HQ response was associated with chromatid breaks, chromosomal gaps and breaks, and total aberrations other than gaps. The responsiveness of *t,t*-MA showed associations with chromosomal gaps. Similar to *t,t*-MA, a high 1,4-BQ–Alb adduct response was associated with chromosomal gaps. BO–Alb adduct level was not predictive of any type of chromosomal aberration.

Correlations Between Urinary Metabolites, Albumin Adducts, and Blood Cell Counts

Correlations between the blood cell counts and the urinary metabolites or albumin adducts were significant, although relatively low (Table 36). An additional set of analyses was performed to determine whether levels of the urinary metabolites were predictive of depressed hematopoiesis above and beyond the amount of depression predicted by benzene exposure itself. The urinary metabolite variables chosen for possible inclusion in the regression

Table 31. Chromosomal Aberrations and Aneuploidy Grouped by Subject According to Benzene Exposure Duration[a]

Variable	Unexposed	Exposure Duration (years)			
		<5	5 to <10	10 to <18	≥18
Number of subjects	51	44	38	28	20
Female (%)	53	39	50	68	65
Smoker (%)[b]	31	45	45	25	30
Age (years)	33.3±7.4	32.2±9.5	38.8±5.8	36.5±4.3	40.0±5.6
Benzene exposure					
Lifetime cumulative (ppm-years)	0	78±86	84±82	77±122	90±55
4-Week mean (ppm)	0.004± 0.003	14.5±16.7	16.2±34.6	3.1±4.0	5.0±5.9
Intensity (ppm/year)	0	28.2±40.2	13.3±13.0	6.4±10.5	4.6±2.9
Total chromatid aberrations (except gaps)[c]	1.20±1.4	1.74±1.5	1.79±1.3	2.27±1.6	2.65±1.7
Total chromosomal aberrations (except gaps)[d]	0.59±1.25	0.95±1.19	0.87±1.12	0.96±1.18	1.15±1.18
Total aberrations (except gaps)[c]	1.78± 2.05	2.70±2.09	2.66 ±1.95	3.23±2.05	3.80±2.44
Chromatid gaps[c]	1.4±1.7	1.3±2.0	0.61±1.1	0.62±0.75	0.60±1.5
Chromatid breaks[c]	1.2±1.4	1.7±1.5	1.8±1.3	2.3±1.6	2.6±1.6
Chromosomal breaks	0.20± 0.49	0.30±0.64	0.29±0.57	0.35± 0.56	0.20±0.52
Acentric fragments[e]	0.39± 0.98	0.51±0.74	0.58±0.86	0.62± 0.98	0.95±1.1
Hypodiploidy (45 chromosomes)	0.22± 0.54	0.21±0.56	0.32±0.70	0.31± 0.55	0
Hypodiploidy (≤44 chromosomes)[c]	2.7± 2.7	4.5±5.9	9.1±8.9	6.6± 7.9	6.7± 5.5
Hyperdiploidy (≥47 chromosomes)	0.06± 0.24	0.14±0.35	0.11±0.31	0.15± 0.46	0.15±0.67
Aneuploidy (45 or ≥47 chromosomes)	0.27± 0.57	0.35±0.72	0.42±0.72	0.46± 0.76	0.15±0.67
Total aneuploidy (≤45 and ≥47 chromosomes)[c]	3.0± 2.7	4.8±5.9	9.5±8.9	7.1± 8.0	6.9±5.4

[a] Unless otherwise noted, the values are reported as means (±SD); chromosomal aberrations and ploidy variables are number per 100 cells; the statistical tests were performed on square-root transformed data. The exposure-response trend was tested by an ANOVA linear contrast which did not control for other factors.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

[c] $P \leq 0.001$, test for exposure-response trend.

[d] $P \leq 0.05$, test for exposure-response trend.

[e] $P \leq 0.01$, test for exposure-response trend.

models were *t,t*-MA, *S*-PMA, and phenol (all as measured after work). For RBCs, WBCs, neutrophils, monocytes, basophils, and platelets, none of the urinary metabolites was predictive of their depressed counts (all $P > 0.10$). In contrast, high *t,t*-MA excretion was predictive of higher lymphocyte counts (not shown).

Correlations Between Blood Cell Counts and Chromosomal Aberrations

The Spearman rank order correlation analyses indicated that there were either weak negative correlations or no associations of blood cell counts with chromosomal aberrations (Table 37). In addition, we performed a set of multiple regression analyses to determine whether the chromosomal aberration and aneuploidy induced by benzene might be accentuated among those who had depressed RBCs and neutrophils. Analyses of the chromosomal aberration and aneuploidy endpoint were undertaken that included both unexposed subjects and benzene-exposed subjects who either had RBC counts below 400 (which was the 18th percentile in the unexposed group and the 49th percentile in the exposed group) or had neutrophil counts below 3000 (which was the 17th percentile in the unexposed group and the 48th percentile in the exposed group).

INTERACTIONS BETWEEN BENZENE EXPOSURE AND OTHER FACTORS

Interaction analyses based on multiple regression procedures were performed to evaluate several possibilities of differences in individual susceptibility or the coacting effects of environmental exposure to another substance besides benzene. The interaction term was the product of

Table 32. Chromosomal Aberrations and Aneuploidy Grouped by Subject According to Benzene Exposure Intensity[a]

Variable	Unexposed	Exposure Intensity (ppm/years)			
		> 0 to < 5	5 to < 15	15 to < 40	≥ 40
Number of subjects	51	54	36	29	11
Female (%)	53	54	53	48	55
Smoker (%)[b]	31	35	42	45	27
Age (years)	33.3 ± 7.4	35.2 ± 7.6	39.3 ± 5.2	36.3 ± 8.7	31.4 ± 8.6
Benzene exposure					
Lifetime cumulative (ppm-years)	0	32 ± 21	74 ± 51	123 ± 65	237 ± 188
4-Week mean (ppm)	0.004 ± 0.003	3.07 ± 2.9	5.89 ± 4.8	17.4 ± 15.5	50.6 ± 55.4
Duration (years)	0	11.2 ± 6.4	9.8 ± 6.0	4.4 ± 1.9	4.0 ± 3.2
Total chromatid aberrations (except gaps)[c]	1.20 ± 1.4	1.89 ± 1.4	2.23 ± 1.6	1.89 ± 1.6	2.18 ± 1.7
Total chromosomal aberrations (except gaps)[d]	0.59 ± 1.3	0.85 ± 1.0	1.00 ± 1.4	1.14 ± 1.1	0.91 ± 1.2
Total aberrations (except gaps)[c]	1.78 ± 2.14	2.74 ± 1.95	3.23 ± 2.25	3.03 ± 2.10	3.09 ± 2.63
Chromatid gaps	1.4 ± 1.7	0.58 ± 1.2	0.37 ± 0.88	1.3 ± 1.9	2.6 ± 1.7
Chromatid breaks[c]	1.2 ± 1.4	1.9 ± 1.4	2.2 ± 1.6	1.9 ± 1.6	2.2 ± 1.7
Chromosomal breaks[d]	0.20 ± 0.49	0.15 ± 0.36	0.43 ± 0.78	0.32 ± 0.55	0.45 ± 0.69
Hypodiploidy (45 chromosomes)[d]	0.22 ± 0.54	0.09 ± 0.30	0.14 ± 0.36	0.50 ± 0.92	0.45 ± 0.69
Hypodiploidy (≤44 chromosomes)[d]	2.7 ± 2.7	6.7 ± 7.0	7.1 ± 6.7	7.0 ± 9.7	3.4 ± 3.6
Hyperdiploidy (≥47 chromosomes)	0.06 ± 0.24	0.09 ± 0.35	0.23 ± 0.60	0.07 ± 0.26	0.18 ± 0.40
Aneuploidy (45 or ≥47 chromosomes)[c]	0.27 ± 0.57	0.19 ± 0.52	0.37 ± 0.65	0.57 ± 1.03	0.64 ± 0.67
Total aneuploidy (≤45 and ≥47 chromosomes)[c]	3.0 ± 2.7	6.9 ± 7.1	7.5 ± 6.7	7.6 ± 9.6	4.0 ± 4.0
Metaphases/1000 cells[d]	45 ± 26	48 ± 25	49 ± 25	53 ± 24	65 ± 30

[a] Unless otherwise noted, the values are reported as means (± SD); chromosomal aberrations and ploidy variables are number per 100 cells; the statistical tests were performed on square-root–transformed data. The exposure-response trend was tested by an ANOVA linear contrast which did not control for other factors.

[b] Includes self-reported smokers and subjects with cotinine levels >100 μg/g creatinine.

[c] $P \leq 0.01$, test for exposure-response trend.

[d] $P \leq 0.05$, test for exposure-response trend.

the dichotomous sex, smoking, or toluene exposure variable times the continuous benzene exposure variable. (The geometric mean of toluene concentrations over 4 weeks of monitoring was dichotomized at 20 ppm.) In these analyses the main effects for exposure or susceptibility variables were first included in the analysis, then the interaction term with benzene was added. Other possible confounding variables such as sex, smoking, or toluene exposure were also entered into the model.

Interactions with Sex

One question of interest is whether men and women differ in the relative efficiencies of their benzene metabolic pathways, which would imply that for a given exposure one sex would excrete more of (or more rapidly) a particular benzene metabolite. There might then also be differences in the degree of damage caused by the metabolite (or intermediates in its pathway), which could manifest in the blood or chromosomal measures.

Interactions between sex and benzene exposure had significant effects on urinary metabolites (Table 38). Without exception, women metabolized benzene more efficiently than men. However, there were no statistically significant interactions of sex with benzene exposure for albumin adducts, blood cell counts, or chromosomal aberrations except for grouped category of total chromosomal aberrations. Of the 23 blood variables examined (included in Table 39; stimulated cells/1000 cells and metaphases/1000 cells were not included in this analysis), only 1 showed a statistically significant interaction, an incidence (1/23) consistent with chance (data not shown).

Interactions with Smoking

The interactive effects of smoking were examined both across the entire exposure range and among only those subjects with relatively low exposures, below 5 ppm

Table 33. Spearman Rank Order Correlation of Urinary Metabolites with Albumin Adducts

Metabolite[a]	Adduct	
	BO–Alb	1,4-BQ–Alb
S-PMA		
Before work	0.68	0.67
After work	0.75	0.69
After − before	0.37	0.28
t,t-MA		
Before work	0.72	0.69
After work	0.75	0.75
After − before	0.50	0.50
Phenol		
Before work	0.21	0.34
After work	0.48	0.46
After − before	0.35	0.24
HQ (phase 1 data only)		
After work	0.46[b]	0.35
CAT (phase 1 data only)		
After work	0.54[c]	0.37

[a] For *S*-PMA, *t,t*-MA, and phenol, a correlation of approximately of 0.13 to 0.15 is statistically significant, depending on the amount of missing data.

[b] $P = 0.02$.

[c] $P = 0.005$.

Table 35. Spearman Rank Order Correlation of Chromosomal Aberrations with the Responsiveness of Albumin Adducts and the Urinary Metabolites HQ and *t,t*-MA[a]

Chromosomal Aberrations	Adduct Residuals		Metabolite Residuals	
	BO–Alb	1,4-BQ–Alb	HQ	*t,t*-MA
Total aberrations (except gaps)	0.04	−0.01	0.47[b]	−0.02
Chromatid gaps	0.12	0.05	0.18	−0.01
Chromatid breaks	0.01	−0.04	0.44[c]	−0.06
Chromosomal gaps	0.13	0.16	0.30[d]	0.22[e]
Chromosomal breaks	0.09	0.09	0.33[f]	0.06
Acentric fragments	−0.02	0.03	0.02	0.02
Hypoploidy	0.11	0.06	0.03	0.08
Total aneuploidy	0.07	−0.02	−0.02	−0.02

[a] The 4-week mean benzene exposure level was regressed on albumin adducts and the current-day benzene exposure level was regressed on urinary metabolites; then the studentized residuals were calculated for each observation. These residuals indicated whether they showed greater than or less than expected response to the benzene exposure. The residuals were then correlated with the chromosomal aberration outcomes.

[b] $P = 0.001$ for Spearman rank order correlations.

[c] $P = 0.002$ for Spearman rank order correlations.

[d] $P = 0.04$ for Spearman rank order correlations.

[e] $P = 0.007$ for Spearman rank order correlations.

[f] $P = 0.02$ for Spearman rank order correlations.

Table 34. Spearman Rank Order Correlation of Urinary Metabolites or Albumin Adducts with Chromosomal Aberrations[a]

Metabolite or Adduct	Chromosomal Aberrations			
	Chromatid Gaps	Chromatid Breaks	Total Aberrations	Total Aneuploidy
S-PMA, after work	−0.13	0.18	0.16	0.05
t,t-MA, after work	−0.05	0.13	0.15	0.08
Phenol, after work	0.16	−0.03	0.01	0.15
BO–Alb	0.00	0.23	0.24	0.12
1,4-BQ–Alb	0.06	0.10	0.12	0.07

[a] A correlation of approximately 0.13 to 0.15 is statistically significant, depending on the amount of missing data.

Table 36. Spearman Rank Order Correlation of Urinary Metabolites or Albumin Adducts with Blood Cell Counts[a]

Metabolite or Adduct	Blood Cell Counts		
	Red Blood Cells	White Blood Cells	Neutrophils
S-PMA, after work	−0.31	−0.17	−0.22
t,t-MA, after work	−0.26	−0.14	−0.23
Phenol, after work	−0.12	−0.19	−0.19
BO–Alb	−0.28	−0.23	−0.25
1,4-BQ–Alb	−0.17	−0.19	−0.27

[a] A correlation of approximately –0.13 to –0.15 is statistically significant, depending on the amount of missing data.

Table 37. Spearman Rank Order Correlation of Chromosomal Aberrations with Blood Cell Counts[a]

Chromosomal Aberrations	Blood Cell Counts		
	Red Blood Cells	White Blood Cells	Neutrophils
Total chromatid aberrations (except gaps)	−0.23	−0.18	−0.15
Total chromosomal aberrations (except gaps)	−0.11	−0.17	−0.11
Total aberrations (except gaps)	−0.23	−0.22	−0.14
Aneuploidy	−0.08	−0.08	−0.02

[a] A correlation of approximately –0.13 to –0.15 is statistically significant, depending on the amount of missing data.

(Table 39). The results are difficult to interpret because the direction of the interactions was almost evenly divided between a greater effect among smokers and a greater effect among nonsmokers. Furthermore, there was little consistency between the results across the entire exposure range and the corresponding results in the lower exposure range only. None of the interactive effects for blood cell counts or chromosomal variables reached a conventional level of statistical significance ($P \leq 0.05$). We conclude that there were probably no meaningful interactive effects of smoking and benzene exposure.

Table 38. Interactions Between Sex and Benzene Exposure on Urinary Metabolites or Albumin Adducts[a]

Metabolite or Adduct	*P* Value for Interaction with Sex[b]	Benzene Regression for Men[c]	Benzene Regression for Women[c]
S-PMA			
Before work	F; <0.0001	0.014±0.011	0.077±0.012
After work	F; <0.0001	0.054±0.012	0.104±0.012
After − before	—	—	—
t,t-MA			
Before work	F; <0.0001	0.011±0.003	0.038±0.004
After work	F; <0.0001	0.023±0.006	0.056±0.006
After − before	F; 0.08	0.013±0.005	0.018±0.005
Phenol			
Before work	F; 0.0002	−0.002±0.011	0.044±0.011
After work	F; 0.008	0.046±0 .011	0.083±0.010
After − before	—	—	—
BO-Alb adducts	—	—	—
1,4-BQ–Alb adducts	F; 0.06	0.022 ±0.003	0.029±0.003

[a] Data used for analysis were all log-transformed; they were adjusted for exposure level and sex main effects, and optionally for toluene and cotinine effects. The analyses were conducted by least-squares regression.

[b] F = females had a greater effect. Only *P* values ≤ 0.10 for the interaction terms are shown.

[c] Values shown are regression coefficients ± SE.

Interactions with Toluene Exposure

High toluene levels have been reported to competitively inhibit benzene metabolism (Inoue et al 1988a), so it was important to examine whether there were interactions between the 2 exposures in relation to the levels of biomarkers. We first simultaneously tabulated the outcome variables according to high or low benzene exposure and high or low toluene exposure (Table 40). Then we evaluated whether there were any interactions between toluene exposure level (dichotomized) and benzene effects in a regression analysis (Appendix B, Table B.12). Only 5 of 37 variables analyzed showed evidence of an interaction at a conventional level of statistical significance ($P \leq 0.05$), and there did not seem to be any consistent pattern of the high-toluene group or the low-toluene group showing a greater effect. Therefore, we conclude there was no important interaction between benzene and toluene exposures in conferring risk. This conclusion, together with the assessment that toluene did not serve as a confounder of benzene effects (Table 28 and Appendix B, Table B.1), led us to believe that toluene exposure did not play an appreciable role in this study.

Table 39. Interactions Between Smoking and Benzene Exposure on Blood Cell Counts, Urinary Metabolites, Albumin Adducts, Chromosomal Aberrations, and Aneuploidy[a]

Variable[b]	Interaction with Smoking[c]	Interaction Between Low Exposure and Smoking[c,d]	Benzene Regression for Nonsmokers[e]	Benzene Regression for Smokers[e]
Red blood cells ($\times 10^{10}$/L)	—	—	—	—
Hematocrit	—	—	—	—
Platelets ($\times 10^{9}$/L)	—	—	—	—
White blood cells ($\times 10^{6}$/L)	—	—	—	—
Lymphocytes ($\times 10^{6}$/L)	—	—	—	—
Neutrophils ($\times 10^{6}$/L)	—	—	—	—
Monocytes, log($\times 10^{6}$/L)	—	—	—	—
Eosinophils, log($\times 10^{6}$/L)	—	—	—	—
Basophils, log($\times 10^{6}$/L)	—	—	—	—
Band cells($\sqrt{}$)	—	—	—	—
Atypical lymphocytes ($\sqrt{}$)	—	—	—	—
S-PMA (log µg/g creatinine)				
Before work	—	—	—	—
After work	—	—	—	—
After − before	—	NS; 0.08	0.032 ± 0.007	0.038 ± 0.012
t,t-MA (log mg/g creatinine)				
Before work	NS; 0.04	—	0.032 ± 0.003	0.009 ± 0.006
After work	—	S; 0.08	0.046 ± 0.005	0.028 ± 0.008
After − before	—	—	—	—
Phenol (log mg/g creatinine)				
Before work	—	—	—	—
After work	—	—	—	—
After − before	NS; 0.003	—	3.02 ± 0.32	0.68 ± 0.35
t,t-MA/*S*-PMA ratio (log)	—	—		
BO–Alb adducts, log(nmol/g albumin)	—	—	—	—
1,4-BQ–Alb adducts, log(nmol/g albumin)	NS; 0.05	—	0.029 ± 0.003	0.021 ± 0.004
Total chromatid aberrations (except gaps) ($\sqrt{}$)	S; 0.1	—	−0.002 ± 0.004	0.010 ± 0.006
Total chromosomal aberrations (except gaps) ($\sqrt{}$)	—	—	—	—
Total aberrations (except gaps) ($\sqrt{}$)	S; 0.05	—	0.0001 ± 0.005	0.011 ± 0.006
Chromatid gaps ($\sqrt{}$)	S; 0.10	S; 0.03	0.012 ± 0.004	0.004 ± 0.006
Chromatid breaks ($\sqrt{}$)	S; 0.06	—	−0.002 ± 0.004	0.010 ± 0.006
Chromosomal breaks ($\sqrt{}$)	—	—	—	—
Acentric fragments ($\sqrt{}$)	—	—	—	—
Hypodiploidy (45 chromosomes)($\sqrt{}$)	NS; 0.08	—	0.006 ± 0.002	−0.002 ± 0.003
Hypodiploidy(≤44 chromosomes) ($\sqrt{}$)	—	—	—	—
Hyperdiploidy (≥47 chromosomes) ($\sqrt{}$)	—	—	—	—
Aneuploidy (45 or ≥47 chromosomes) ($\sqrt{}$)	—	—	—	—
Total aneuploidy (≤45 and ≥47 chromosomes) ($\sqrt{}$)	—	—	—	—
Stimulated cells/1000 cells[f]	—	—	—	—
Metaphases/1000 cells	—	—	—	—

[a] Data were adjusted for exposure level and smoking main effects, and optionally for age and toluene effects. All subjects were included in the analyses; control subjects were randomly assigned values between 0 and 0.01 ppm for benzene exposure, which was the approximately lowest detection limit. Interaction analyses are also presented for a subset of subjects with low benzene exposure (≤ 5 ppm). The analyses were conducted by least-squares regression. Dashes indicate $P > 0.10$.

[b] ($\sqrt{}$) = square root transformation using the square root of (x + 3/8), where x is the raw variable.

[c] *S* and *NS* indicate whether smokers or nonsmokers had the greater effect.

[d] This comparison of regession slopes for smokers and nonsmokers includes only those subjects with benzene exposure ≤5ppm.

[e] Values shown are regression coefficients ± SE.

[f] Lymphocytes that have been stimulated to divide using phytohemagglutinin per 1000 cells evaluated.

Table 40. Blood Cell Counts, Urinary Metabolites, Albumin Adducts, Chromosomal Aberrations, and Aneuploidy by Benzene and Toluene Exposure Levels[a]

Variable	Unexposed	Exposure Level			
		Low Benzene, Low Toluene	Low Benzene, High Toluene	High Benzene, Low Toluene	High Benzene, High Toluene
Number of subjects	51	45	28	27	30
Female (%)	53	64	39	56	43
Smoker (%)[b]	31	29	46	33	50
Age (years)	33.3±7.4	35.9±6.0	37.6±7.1	32.7±8.9	38.7±8.1
Benzene exposure					
Lifetime cumulative (ppm-years)	0	46±27	35±23	186±135	82±58
Duration (years)	0	10.3±6.0	7.7±5.3	5.0±3.5	10.4±7.3
4-Week mean (ppm)	0.004±0.003	1.8±1.3	3.0±1.1	28.1±16.6	12.3±10.6
Toluene exposure, 4-week geometric mean ± GSD (ppm)	0.004±0.003	13.3±4.8	28.7±12.9	4.4±4.4	46.3±40.8
Red blood cells ($\times 10^{10}$/L)	463±52	392±58	412±60	400±44	404±61
Hematocrit (phase 2 only)	44.2±5.3	42.9±3.5	43.0±3.6	42.5±3.5	43.0±5.1
Platelets ($\times 10^{9}$/L; phase 2 only)	277±43	276±65	287±57	244±11	284±79
White blood cells ($\times 10^{6}$/L)	6671±1502	6671±1502	6364±1311	5211±955	6130±1823
Lymphocytes ($\times 10^{6}$/L)	2205±789	2205±789	2422±741	1970±502	2284±852
Neutrophils ($\times 10^{6}$/L)	4006±1108	3498±921	3609±993	2928±757	3311±1408
Monocytes ($\times 10^{6}$/L)	267±139	298±145	282±144	204±107	315±162
Eosinophils ($\times 10^{6}$/L)	145±162	102±91	123±105	72±57	183±190
Basophils ($\times 10^{6}$/L)	9.3±19.3	10.7±15.5	3.0±8.0	4.3±9.9	10.2±17.9
Band cells	32.6±44.1	34±32	33±52	30±40	23±37
Atypical lymphocytes	0.10±0.22	0.17±0.35	0.34±0.48	0.04±0.14	0.09±0.21
S-PMA (µg/g creatinine)					
Before work	1.8±4.3	46±67	40±58	113±152	148±239
After work	1.9±1.9	98±140	84±124	410±384	643±1195
After − before	0.07±4.5	51±129	44±113	297±361	495±1063
t,t-MA (mg/g creatinine)					
Before work	0.34±0.57	0.66±0.67	0.95±0.56	5.3±6.9	2.2±2.0
After work	0.26±0.27	2.1±2.6	3.3±2.5	14.2±7.3	7.7±5.2
After − before	−0.08±0.62	1.5±2.6	2.4±2.5	8.8±7.1	5.4±4.1
Phenol (mg/g creatinine)					
Before work	11.7±21	8.5±23	8.9±14	33±65	13±18
After work	6.9±10.0	12.4±15.7	9.5±9.5	90±98	23±26
After − before	−4.9±21	3.9±18	0.7±17	60±117	10±30
t,t-MA/*S*-PMA ratio (log)	0.23±0.25	0.06±0.06	0.12±0.14	0.07±0.10	0.05±0.06
Total chromatid aberrations (except gaps) ($\sqrt{}$)	1.2±1.4	2.2±1.6	1.6±1.4	2.3±1.6	1.8±1.4
Total chromosomal aberrations (except gaps) ($\sqrt{}$)	0.59±1.3	1.11±1.3	0.75±1.0	1.15±1.2	0.75±1.1
Total aberrations (except gaps) ($\sqrt{}$)	1.8±2.1	3.4±2.1	2.4±1.9	3.4±2.1	2.5±2.1
Chromatid gaps ($\sqrt{}$)	1.4±1.7	0.7±1.1	0.4±.8	2.3±2.0	0.3±1.1
Chromatid breaks ($\sqrt{}$)	1.1±1.3	2.2±1.5	1.6±1.4	2.3±1.6	1.8±1.4
Chromosomal breaks ($\sqrt{}$)	0.20±0.49	0.33±0.64	0.11±0.32	0.42±0.64	0.29±0.60
Acentric fragments ($\sqrt{}$)	0.39±0.98	0.73±0.99	0.64±0.99	0.58±0.81	0.46±0.69
Hypodiploidy (45 chromosomes)($\sqrt{}$)	0.22±0.54	0.16±0.37	0.07±0.26	0.65±0.85	0.11±0.57
Hypodiploidy(≤44 chromosomes) ($\sqrt{}$)	2.7±2.7	6.8±7.7	7.3±6.2	2.6±2.5	9.5±9.6
Hyperdiploidy (≥47 chromosomes) ($\sqrt{}$)	0.06±0.24	0.20±0.59	0.11±0.32	0.15±0.37	0.04±0.19
Aneuploidy (45 or ≥47 chromosomes) ($\sqrt{}$)	0.27±0.57	0.36±0.71	0.18±0.39	0.81±0.94	0.14±0.59
Total aneuploidy (≤45 and ≥47 chromosomes) ($\sqrt{}$)	3.0±2.7	7.2±7.8	7.4±6.2	3.4±3.1	9.6±9.5
Stimulated cells/1000 cells[f]	568±126	592±142	564±125	604±113	557±138
Metaphases/1000 cells	45±26	46±23	54±28	64±22	44±25

[a] A high benzene level was defined as > 5 ppm and a high toluene level was defined as a geometric mean > 20 ppm. Unless otherwise specified, values are presented as arithmetic means ± SD.

[b] Includes self-reported smokers and subjects with cotinine levels >100 µg/g creatinine.

DISCUSSION

Benzene is a well-recognized hematotoxic agent and human leukemogen; however, whether exposure to low levels of benzene constitutes a risk of adverse health effects remains unknown. Increasing public concern over the possibility of such a risk has resulted in a call for more studies using specific and sensitive biomarkers to assess the effects of benzene exposure at low levels. This project is one of several studies designed to validate biomarkers of benzene exposure in humans (Ong et al 1995, 1996; Pezzagno et al 1999). The participating subjects in the present study were recruited from Chinese occupational populations with a broad range of benzene exposures. This international multilaboratory collaboration provided a unique opportunity to evaluate the relation between benzene exposure and different biomarkers.

The entire study was conducted under a strict Quality Assurance Plan. Standard operating procedures were drafted and documented before the study began and were followed in all steps involved. Work both in the field and in the laboratories was audited and investigated by a third party (Arthur D Little) to ensure the quality of the data generated from this study (Appendix C).

BENZENE EXPOSURE

For the present study to validate biomarkers of benzene exposure, the most important and critical issue was that the personal exposure data be valid. There are always reasons to question the validity of the exposure data collected in field studies because a number of known factors may greatly affect the ultimate results of exposure levels. For example, personal samplers might be contaminated in the field during sampling, or the amount of benzene obtained at sampling might decrease during storage and shipment of the samples. In order to examine the validity of the exposure data in this study, we incorporated several quality control procedures in the processes of sampling and analysis of benzene.

First, the stability of benzene in the samplers was investigated at room temperature for up to 25 days in the NYU laboratory before sampling in the field. The results indicated that benzene was stable enough in the samplers to withstand a period of storage and shipment after sampling (Table 2). Second, the measurements of benzene in blank samples prepared in the field were all below the detection limit, indicating that there was no appreciable benzene contamination of our samples. In addition, benzene recovery results in spiked samples analyzed after storage and shipment were similar to those in the samples of the stability study conducted at NYU (Table 2). These results indicated that there was no significant loss of benzene from individual personal samplers during the 1-month period of storage and shipment. Furthermore, the quality control procedures for GC analysis of benzene proved that benzene exposure levels reported in this study were accurate with very small variations and therefore reflected actual exposure levels for all participating study subjects (Table 3). Best of all, the benzene recovery results in parallel samples analyzed in Beijing, China, and at NYU showed excellent agreement (Figure 5), suggesting that our exposure data were valid and suitable for evaluation of the selected biomarkers.

BLOOD CELL COUNTS

It has long been recognized that exposure to high levels of benzene can result in a depression of blood cell counts (Goldstein 1977, 1988; Aksoy 1988b; Kipen et al 1989; Khuder et al 1999). Significant decreases of WBC, RBCs, and platelets have been observed in human populations exposed to relatively high levels of benzene (Hernberg et al 1966; Aksoy et al 1971, 1987). However, few studies have attempted to examine the relation between benzene exposure and hematologic response over a broad and well-characterized range of exposures. From the limited published data on the exposure-response relation, Ward and associates (1996) indicated that blood cell depression was unlikely to occur at levels below 10 ppm. More recently, however, Khuder and coworkers (1999) reported decreases in absolute RBC and platelet counts in workers followed longitudinally while exposed to relatively low levels of benzene (mean levels between 0.14 and 2.08 ppm).

The initial data analyses of our present study indicated that both WBCs and RBCs declined in an exposure-dependent pattern in this population with occupational exposures to benzene ranging from 0.06 to 122 ppm (Figure 8). Surprisingly, further analysis indicated that RBCs and WBCs were significantly lower even in the 16 subjects of the lowest benzene exposure group (4-week mean concentration at or below 0.5 ppm) when compared with the unexposed subjects (Table 7). The 4-week mean benzene concentration we used may not be the most appropriate measure for correlation analysis with blood cell counts, however, because these values may reflect the effects of more extended benzene exposure. Therefore, we calculated the corresponding individual cumulative exposure duration (in years) and exposure intensity (mean exposure level per year) for this lowest exposure group. For these workers, the median exposure duration was 14 years and the median exposure intensity was 2.68 ppm. These findings suggest that chronic exposure to benzene at low levels for prolonged periods may result in hematologic suppression in humans.

As stated by British Industrial Health Board (cited in Goldwater 1941), "while the most characteristic blood picture in benzol poisoning remains that described by the earlier authors, a leucopenia with neutropenia, thrombopenia and some anemia, it becomes increasingly evident that many cases present wide variations on this picture." Similarly, several investigators have reported a variety of hematologic changes (or lack of effects) in benzene-exposed workers (for overview, see Askoy 1988b; Bogadi-Sare et al 1995; Conservation of Clean Air and Water in Western Europe [CONCAWE] 1996). The decrease in WBCs and neutrophils seen in our studies is consistent with earlier reports of hematologic changes in benzene-exposed workers (Aksoy 1988b). Recently, some animal and human studies have shown that within the WBCs, lymphocytes appear to be more sensitive than other cell types (Ward et al 1985; Rothman et al 1996). However, the selective effect of benzene on lymphocytes has not been as clearly documented in humans (Ward et al 1996).

A few studies have shown discrepancies in the effect of benzene exposure on peripheral lymphocyte counts (Rothman et al 1996; Ward et al 1996). Rothman and colleagues (1996) compared hematologic outcomes in a cross-sectional study of 44 workers heavily exposed to benzene (median exposure, 31 ppm) and an unexposed control population of 44 Chinese workers matched for age and sex. They observed that WBCs, absolute lymphocyte count, platelets, and RBCs all were significantly decreased among the exposed workers compared with the unexposed controls. In a subgroup of workers who were not exposed to benzene concentrations higher than 31 ppm on any of 5 sampling days, only the absolute lymphocyte counts differed significantly between exposed workers and control subjects. These authors concluded that the absolute lymphocyte count is the most sensitive indicator of benzene-associated hematotoxicity. Ward and associates (1996) analyzed hematologic screening data collected over a 35-year period at a rubber hydrochloride manufacturing plant to examine the relation between benzene exposure and hematologic parameters. They observed that both WBCs and RBCs significantly decreased with elevated levels of benzene exposure. Their data also showed a stronger effect of benzene on WBCs than on RBCs but did not provide evidence that low WBC counts were due to selective depletion of lymphocytes.

In our present study, the regression analyses of lymphocytes by benzene exposure (both 4-week mean concentration and cumulative exposure intensity), with adjustment for sex, age, cotinine level, and toluene exposure, indicated that the lymphocyte depression was indeed associated with benzene exposure. The association was not as strong as Rothman's findings, however, and the lymphocytes were not found to be more sensitive to benzene exposure than were other cell types (Appendix B, Tables B.1 and B.2).

Methodologic differences in counting lymphocytes may have contributed to differences in the findings of benzene-associated lymphocyte depression reported in different studies. The absolute lymphocyte counts were derived from a manual count of 100 cells per slide in Ward and associates' study (1996), while in our study 3 sets of slides totaling 900 cells were manually counted for differentiation. Rothman and colleagues (1996) counted the lymphocytes using an automated differential cell counter. A number of studies (Goldstein 1988; LaMontagne et al 1993) indicated high variability in the WBC differentials obtained by manual counting of only 100 WBCs. Consistent with this observation, Rothman and colleagues (1996) also performed a standard manual count of 100 cells to obtain absolute lymphocyte counts and found similar numbers for exposed and unexposed workers. Therefore, it is recommended that at least 400 WBCs be evaluated when manual counts are to be used.

Unlike the findings reported by Rothman and colleagues (1996), the present study showed clear-cut exposure-response relations for neutrophils whether the regression analyses were conducted with or without adjustment for confounding factors. This exposure-dependent decrease of neutrophils suggests that the low WBC counts were due to a selective effect of benzene on neutrophils.

URINARY METABOLITES

This study sought to examine the relation between benzene exposure level and several different urinary metabolites and to determine how well each metabolite could reflect personal benzene exposure, as well as to evaluate these metabolites' relations with albumin adducts and chromosomal aberrations. The major metabolites found in urine after exposure to benzene are phenol and its conjugates (Yardley-Jones et al 1991). Therefore, phenol has long been used as a biomarker for occupational exposure to benzene. However, its application in populations with low levels of benzene exposure has been questioned because a substantial background level of phenol has been found in unexposed subjects due to sources other than benzene exposure (Ong and Lee 1994).

Consequently, other metabolites of benzene have been proposed to replace phenol as a biomarker, including *S*-PMA, *t,t*-MA, HQ, CAT, and BT (Inoue et al 1989a,b; Stommel et al 1989; Lee et al 1993; van Sittert et al 1993; Ong et al 1995). An HEI-sponsored project (Melikian et al 1999a) established improved LC-MS/MS methods for analyzing many of these metabolites. The suitability of these

metabolites to serve as biomarkers in human populations in terms of specificity, sensitivity, and reproducibility needed to be further validated. Before the new LC-MS/MS methods were developed, a number of evaluations of these metabolite biomarkers had been conducted in the field (Boogaard and van Sittert 1995, 1996; Ghittori et al 1995, 1999; Ong et al 1996; Hotz et al 1997; Kivisto et al 1997, Pezzagno et al 1999). In phase 1 of the present study (high levels of exposure), we evaluated the potential applications and predictive abilities of 6 urinary benzene metabolites, *t,t*-MA, *S*-PMA, BT, HQ, CAT, and phenol, as biomarkers of exposure to benzene in humans (Qu et al 2000). *S*-PMA and *t,t*-MA were observed to be most sensitive among the 6 biomarkers. Therefore, only *S*-PMA, *t,t*-MA, and phenol were further examined for their relations with a broad range of benzene exposures in phase 2 of the study.

As previously noted, to ensure that the levels of urinary metabolites measured were reproducible with the methods employed, 10 quality control samples were prepared from each of the 2 separate pools of urine in phase 1 and 2. The 10 aliquots were coded with a 4-digit random number as if they were regular study samples, so the staff analyzing the samples were blinded to the sample sources and identities. The results showed that the measurements were quite reproducible for all metabolites including BT. It is important to note that the measurement reliability and variability described here is not just duplicate measurements performed in a laboratory run, as variability is commonly assessed, but includes variability across runs in the laboratory; thus it reflects day-to-day variability in calibration, reagents, procedures, etc, and is therefore a more comprehensive assessment of reliability than are the usual duplicate measurements.

S-PMA and *t,t*-MA as Biomarkers for Low-Level Benzene Exposure

S-PMA is a marker for a detoxification pathway in benzene metabolism, and its measurement has been shown to be useful for monitoring subjects exposed to benzene at low ppm levels using different methods (Stommel et al 1989; van Sittert et al 1993; Popp et al 1994). Phase 1 of this study showed that the levels of *S*-PMA in urine collected from most of the unexposed subjects before and after work were below the detectable limit, whereas the levels in exposed subjects were much higher, especially in samples collected after work ($P < 0.0001$, Figure 12). The increases of *S*-PMA levels from before work to after work in exposed subjects were strongly correlated with the current-day benzene exposure levels (Table 10). Analyses of these increases, controlling for age, sex, and smoking status as possible confounders, and then determining whether other exposure measurements (toluene and xylene) added further predictiveness, continued to show strong associations with current-day benzene exposure as a continuous variable. They did not show any confounding by age or smoking. All of this suggests that *S*-PMA is a specific metabolite of benzene and is not likely to be derived from any other common sources. Because the background levels of urinary *S*-PMA in unexposed control subjects were extremely low, and excretion of *S*-PMA was about 400-fold higher in exposed subjects than in unexposed subjects, *S*-PMA appears to be a good biomarker for detecting and evaluating benzene exposure at low levels (Table 12).

From the difference between urinary *S*-PMA levels after work and those before work, we calculated that exposure to 1 ppm benzene would give an increase of 15.3 µg of *S*-PMA/g creatinine over and above the background level (0.88 µg/g creatinine in unexposed subjects, Table 12) at the end of an approximately 8-hour work shift. Therefore, it can be predicted that *S*-PMA would distinguish an unexposed group from a group of subjects exposed to benzene at levels as low as 0.1 ppm. The results in phase 2 of the study further indicated that *S*-PMA correlated very well with personal benzene exposures across a broad range of exposures from 0.06 to 122 ppm. A clear-cut exposure-response relation between benzene exposure and *S*-PMA was observed not only in the total exposed group (Figures 12, 14, and 17) but also in the subgroup with benzene exposure below 1 ppm (Table 16). Further examination indicated that the after-work levels of *S*-PMA in subjects with benzene exposures of 0.5 ppm or lower (in fact, all were < 0.25 ppm) were significantly higher than those in unexposed subjects ($P < 0.003$). This confirms the sensitivity of *S*-PMA calculated in phase 1 of the study.

Less than 2% of absorbed benzene is estimated to be eliminated as *t,t*-MA (Ong et al 1995). However, its formation in the body is believed to be mainly through benzene metabolism with a possible small contribution from metabolism of sorbic acid used as a preservative in food, cosmetics, and other substances; thus *t,t*-MA is fairly specific to benzene exposure (Johnson and Lucier 1992). In the present study, the low background level and relatively small variation from time to time in the unexposed subjects (Figure 15) demonstrated that the contribution to *t,t*-MA formation from other sources was minor. Like *S*-PMA, *t,t*-MA in urine collected after work from exposed subjects increased substantially and dose dependently (Figures 13 and 15). The increases of *t,t*-MA in exposed subjects from before work to after work were also strongly related to the current-day benzene exposure levels (Figure 15). We calculated, from levels of *t,t*-MA before and after work in the exposed subjects in phase 1 of the study, that

exposure to benzene at 1 ppm would result in a *t,t*-MA increase of 0.62 mg/g creatinine over and above the background level in the unexposed subjects (0.31 mg of *t,t*-MA/g creatinine, Table 12). Therefore, *t,t*-MA could be a good marker for benzene exposure at levels around 1 ppm. Consistent with this, phase 2 of the study showed that the after-work levels of *t,t*-MA could well distinguish between unexposed subjects and subjects exposed to benzene at 1 ppm but failed to detect the difference between unexposed subjects and subjects exposed to benzene at or below 0.5 ppm (all were < 0.25 ppm), despite a clear-cut exposure-response trend over the exposure range from 0 to 1 ppm ($P < 0.001$, Table 16). The limitation of *t,t*-MA as a biomarker for benzene exposures below 0.25 ppm might have been due to potentially confounding factors, including cigarette smoking and sorbic acid intake.

Furthermore, in the present study the before-work levels of *S*-PMA and *t,t*-MA were significantly higher in exposed subjects than in unexposed subjects ($P < 0.001$, Figures 12 and 13). This finding suggests that the previous days of benzene exposure may have contributed substantially to the levels of *S*-PMA and *t,t*-MA measured in urine samples. The hypothesis was confirmed by further analysis of these data. As shown in Table 13, the levels of *S*-PMA and *t,t*-MA were significantly higher in samples collected on Friday morning before work (when subjects had had 4 days of continuous exposure before sampling) than in samples collected on Monday morning before work (when subjects had had no occupational benzene exposure for at least 2 days). Therefore, the difference between the 2 levels reflects the contribution to the detected levels of *S*-PMA and *t,t*-MA from recent exposures. For this reason, we suggest that the difference between before-work and after-work levels of *S*-PMA and *t,t*-MA be used as an indicator of current-day benzene exposure.

Boogaard and van Sittert (1995) investigated the excretion of *S*-PMA and *t,t*-MA in a group of workers exposed to benzene at relatively low levels (< 5 ppm) and reported that exposure to 1 ppm benzene (8 hours time-weighted average) led to a mean *S*-PMA excretion of 47 µg/g creatinine and *t,t*-MA excretion of 1.74 mg/g creatinine. Bechtold and coworkers (1991) also reported that in a group of 14 Chinese female workers who were exposed to 4.4 ppm benzene (8 hours time-weighted average), the *t,t*-MA excreted at the end of the shift was 1.4 mg/g creatinine for each 1 ppm of benzene exposure. Their results are in good agreement with our results calculated from the comparable subgroup of workers exposed to benzene concentrations between 1 and 5 ppm (Figure 20, *S*-PMA at 45.7 µg/g creatinine and *t,t*-MA at 1.6 mg/g creatinine, respectively).

Several studies in mice, rats, and humans found that the lower the level of benzene that was administered, the greater the percentage of benzene that was metabolized into *t,t*-MA (Gad-El Karim et al 1985; Henderson et al 1989; Sabourin et al 1989; Witz et al 1990). A similar trend and pattern was found in our study for both *S*-PMA and *t,t*-MA (Figure 18). The urinary *S*-PMA excretions resulting from 1 ppm benzene exposure were 70.2, 45.7, 50.8, 25.6, and 19.1 µg/g creatinine for the subgroups with benzene exposures of ≤ 1 ppm, > 1 to 5 ppm, > 5 to 15 ppm, > 15 to 30 ppm, and > 30 ppm, respectively. The *t,t*-MA excretion rates per 1 ppm of benzene exposure in each of the same subgroups were 2.8, 1.6, 1.02, 0.59, and 0.28 mg/g creatinine. These nonlinear metabolic rates suggest that the enzymes related to benzene metabolism may become saturated at high levels of benzene exposure.

Our analysis found interindividual variation in the biotransformation of inhaled benzene to urinary *S*-PMA. We estimated that, at the end of a shift, the fraction of benzene that had been converted to *S*-PMA ranged between 0.005% and 0.3% (Figure 21). This estimate was based on the assumption that each person inhales about 15 L of air per minute and that only 50% of inhaled benzene is exhaled. Ghittori and associates (1999) reported that the biotransformation of benzene to *S*-PMA in smokers was significantly variable, from 0.01% to 0.21%. Boogaard and van Sittert (1995) also reported that in exposed workers the conversion of benzene to *S*-PMA ranged from 0.05% to 0.26%. In this present study, the most frequent conversion rate was about 0.03% of the inhaled benzene (8-hour sample) or *S*-PMA formation at 12 µg/g creatinine per 1 ppm benzene for all exposure subgroups (Figure 21).

There was also interindividual variation in the conversion rate of benzene into *t,t*-MA at the end of the shift, ranging from 0.6% to approximately 20% of 8-hour inhaled benzene (Figure 22). Unlike *S*-PMA, however, the most frequent conversion rate for *t,t*-MA varied depending on benzene exposure level. In the subgroup with benzene exposures below 5 ppm, the transformation rate of 1.3 mg/g creatinine per 1 ppm benzene, or about 4.1% of inhaled benzene, was frequent (Figure 22). This value decreased to 0.96% of inhaled benzene in the subgroup exposed to greater than 15 ppm benzene (Figure 22). This is in agreement with the finding in animal models that a greater percentage of benzene is metabolized to *t,t*-MA at lower doses of exposure (Gad-El Karim et al 1985; Henderson et al 1989; Witz et al 1990). Similar results also were reported previously in human studies of environmental tobacco smoke (Yu and Weisel 1996).

These observations suggest that the determination of *t,t*-MA level alone may not accurately reflect personal

exposure in workplaces where people are exposed to high concentrations of benzene. In addition, *t,t*-MA may not be a sufficiently specific biomarker of low benzene exposures because it is known that sorbic acid, a food preservative and fungistatic agent, is metabolized to *t,t*-MA and excreted in urine (Ducos et al 1990; Pezzagno and Maestri 1997; Pezzagno et al 1999). The uptake of sorbic acid is about 25 mg/day in the United States (Yu and Weisel 1996) and 6 to 30 mg/day in Europe (Ruppert et al 1997). Biotransformation rates of sorbic acid into *t,t*-MA are estimated to be in the range of 0.05% to 0.51% (Ruppert et al 1997; Pezzagno and Maestri 1997). The interindividual variability of the conversion rates of sorbic acid into *t,t*-MA might constitute another source of uncertainty in the interpretation of urinary levels of *t,t*-MA.

Toluene is a known competitive inhibitor of benzene metabolism, since they are metabolized by the same enzyme (Andrews et al 1977; Sato and Nakajima 1979; Inoue et al 1988a; Brandeau et al 1992). However, in the present study toluene was not found to significantly interfere with benzene in terms of *S*-PMA or *t,t*-MA formation.

Cigarette smoking is known to affect the level of *t,t*-MA in urine. For example, Melikian and coworkers (1994) reported that the mean levels of *t,t*-MA were 0.22 and 0.24 mg/g creatinine for male and female smokers, respectively, versus 0.06 and 0.05 mg/g creatinine for male and female nonsmokers, respectively. Similar findings in a later study (Pezzagno et al 1999) indicated that the mean values of *t,t*-MA were 0.19 and 0.06 mg/g creatinine for smokers and nonsmokers, respectively. However, the results in phase 1 of the current study did not show any confounding by smoking. This discrepancy was probably due to the fact that the contribution to *t,t*-MA from smoking was too small (less than 0.2 mg/g creatinine in studies previously cited) for its confounding effect to have been detected on the extremely high *t,t*-MA levels seen in phase 1 of this study (14.7 mg/g creatinine). Indeed, during phase 2 of the study, smoking was detected to have a weak effect on the conversion of benzene into *t,t*-MA in after-work samples collected from the low-exposure group (< 5 ppm).

Phenol, HQ, CAT, and BT as Biomarkers

Phenol, a major metabolite of benzene, has been widely investigated and well documented as a biomarker for benzene exposure. However, owing to the high and variable background levels from sources other than benzene, urinary phenol is not considered a suitable marker for benzene exposure below 5 ppm (Boogaard and van Sittert 1995). More recently, urinary HQ and CAT have been explored as markers of benzene exposure (Inoue et al 1988b, 1989b; Ong et al 1995, 1996). In one study Inoue and colleagues (1988b) detected high background levels of HQ and CAT in the urine of unexposed subjects; therefore, neither HQ nor CAT enabled distinction of those exposed to benzene at 10 ppm from those without exposure. More recently, Ong and coworkers (1996) reported a good correlation between benzene in air and HQ in urine for benzene concentrations above 0.25 ppm, but found no correlation at all between benzene and CAT concentrations. Phase 1 of this study showed that HQ and CAT were equally well correlated with benzene exposure at the observed levels. However, they may not be good markers of benzene exposure at low levels encountered in ambient air because they are not specific for benzene, and in this study their background levels in unexposed subjects were high relative to the small contribution from low-level exposure to benzene. We also have found no correlation between benzene exposure and urinary BT (Qu et al 2000; this study). It appears that BT was not stable under the conditions used in this study and may have been further oxidized and converted to several products. This hypothesis is currently under investigation.

ALBUMIN ADDUCTS IN BLOOD

Results from this investigation of benzene-related protein adducts confirmed previous findings that levels of BO–Alb and 1,4-BQ–Alb were highly associated with benzene exposure (Bechtold et al 1992a,b; Yeowell-O'Connell et al 1998). In the current study, we also observed for the first time significant effects of age and the interaction of age and exposure on BO–Alb levels (Table 21) and of both sex and age on 1,4-BQ–Alb levels (Table 23). This study showed that smoking was not associated with elevated levels of BO–Alb or 1,4-BQ–Alb. In contrast, the levels of 1,4-BQ–Alb appeared marginally higher in nonsmokers than in smokers when regression analysis of albumin adducts by benzene exposure level was conducted after adjustment for exposure and smoking (Table 39). We noticed that among the Chinese workers in this study, the smokers were almost exclusively male. Second, sex was shown to interact with benzene exposure on the levels of 1,4-BQ–Alb adducts (higher in female subjects, Table 38). Therefore, we hypothesize that the observed effect on 1,4-BQ–Alb adducts in nonsmokers was probably due to the uneven sex distribution among smokers and nonsmokers.

The relatively high levels of BO–Alb and 1,4-BQ–Alb among unexposed subjects also confirmed earlier observations (Yeowell-O'Connell et al 1998). While elevated levels of 1,4-BQ–Alb would be expected among unexposed control subjects owing to the many endogenous and dietary sources of phenol and HQ, the precursors of 1,4-BQ (McDonald et al 1993), the background of BO–Alb is more difficult to rationalize (Yeowell-O'Connell et al 1998; Rappaport and

Yeowell-O'Connell 1999). On the basis of preliminary in vitro results, we speculate that BO–Alb adducts, measured as *S*-phenylcysteine, can also arise from free-radical–mediated processes involving simple aromatic moieties such as benzoic acid, a common dietary constituent (unpublished work). Clearly, more research into the sources of these background adducts is warranted, as is work to identify other benzene-related protein adducts that might have lower background levels.

Despite the high background levels of adducts observed in this study, the trends between BO–Alb and 1,4-BQ–Alb adducts and benzene exposure were easily discerned (Figure 23), even at a median 4-week mean exposure of 3.77 ppm among the 130 exposed subjects. The ability to detect these trends was enhanced by both the wide range of benzene exposures and the modest contributions of the within-subject and assay-related components of variance. In fact, the combined within-subject and assay-related (assay plus injection) variance components contributed only 6% and 14% of the total variation in 1,4-BQ–Alb and BO–Alb, respectively (see Table 18). Certainly the good precision of the adduct measurements, indicated by assay-related CVs of 11% and 19% for 1,4-BQ–Alb and BO–Alb adducts, respectively, contributed to our ability to detect effects of exposure and covariates on adduct levels. Given the relatively long half-lives of these adducts (the half-life for human serum albumin is about 21 days), the within-subject variance component estimated in Table 18 is probably an underestimate of the true value, owing to the likely autocorrelation of adduct levels measured on 3 consecutive Mondays. Nonetheless, these data suggest that levels of BO–Alb and 1,4-BQ–Alb reflect relatively long-term benzene exposure (weeks to months) but are not particularly affected by transient exposures from day to day. Thus these adducts offer promise in evaluating human exposures to benzene at levels well below 10 ppm. The extent to which this promise can be realized obviously depends on further study and, in part, on our ability to sort out the background sources of adducts.

Finally, our data suggest that there was relatively little decay of adduct levels during periods up to 64 hours after exposure. Since the half-life for human serum albumin is about 21 days, this result was anticipated. However, we did observe a slightly larger (negative) Spearman coefficient of −0.1454 for 1,4-BQ–Alb adducts compared with 0.0246 for BO–Alb adducts (neither coefficient was statistically significant). This could point to moderate chemical instability of the BQ adduct compared with that of BO, consistent with our results in rat studies (Troester et al 2000).

CHROMOSOMAL ABERRATIONS

It has been reported that exposure to high levels of benzene causes chromosomal aberrations, including breaks, gaps, and sister chromatid exchanges, as detected by conventional assays (Forni 1979; Major et al 1994; Tompa et al 1994; Turkel and Egeli 1994). More recently, FISH assays have been developed to detect numerical and structural changes of specific chromosomes in both interphase and metaphase spreads (Gray and Pinkel 1992; Rupa et al 1995) and have been successfully adapted to monitor cytogenetic changes in workers exposed to benzene (Zhang et al 1996, 1998; Smith et al 1998). In the first of a series of studies conducted by researchers from the University of California at Berkeley and the US National Cancer Institute (UCB/NCI), Zhang and colleagues (1996) reported that high benzene exposure (> 31 ppm) induced aneuploidy of chromosome 9, with trisomy being the most prevalent form. However, no difference was detected between unexposed subjects and workers exposed to benzene at or below 31 ppm. Furthermore, they observed in the same population that benzene exposure caused increases in translocations between chromosomes 8 and 21 and was associated with significant increases in hyperdiploidy of chromosomes 8 and 21 (Smith et al 1998). In addition, increased aneusomy and long arm deletion of chromosomes 5 and 7 were detected among exposed workers compared with unexposed subjects (Zhang et al 1998). In the UCB/NCI series of studies, the exposure levels of benzene were very high, ranging from 1.6 to 328.5 ppm, with a median of 31 ppm (Rothman et al 1996). Furthermore, most of the chromosomal changes (except those for chromosome 7) in their studies were found in comparisons between the unexposed group and the group of workers exposed to benzene at concentrations higher than 31 ppm. The significant exposure-response trends found in that study were often reported without indication of whether the control subjects and the subjects exposed to benzene at or below 31 ppm differed significantly in personal characteristics such as age or sex.

The current biomarker study was designed to address the reliability of interphase FISH and tandem FISH to detect structural and numerical alterations occurring in benzene-exposed workers. In phase 1 of the study, 2 chromosomes and 3 harvest times were selected for evaluation among highly exposed workers. Chromosome 1 was chosen because the adjacent α-probe and classical-probe for this chromosome were the most reliable, allowing breakage affecting the breakage-sensitive 1cen-1q12 region to be detected in interphase cells. This probe combination was also chosen as a follow-up to the previous studies with the tandem chromosome 1 probes. To optimize the

sensitivity of this assay for breakage, cells were evaluated prior to mitotic stimulation and at 51 hours, an optimal harvest time to detect chromosomal breakage. The chromosome 7 probe was selected on the basis of the previously reported sensitivity of this chromosome to the aneuploidy-inducing effects of benzene.

Cells were evaluated for chromosomal gain and loss prior to stimulation (G_0 lymphocytes) and after 72 hours, the preferred time to detect aneuploidy induced during cell culture. In addition, FISH studies using the tandem chromosome 1 probes and the chromosome 7 probe were performed on the polymorphonuclear cells in the peripheral blood prior to mitotic stimulation. These cells were chosen as they are derived from the cell lineage primarily affected in benzene-induced leukemia (Aksoy 1988a). Unlike the findings of Smith and coworkers, no significant changes either numerical or structural were detected in the cultured lymphocytes of the exposed and unexposed subjects recruited in phase 1 of this study. A minor increase in hyperdiploid cells was seen in the unstimulated G_0 lymphocytes. Interestingly, the frequency of breakage affecting the 1cen-1q12 region of chromosome 1 was actually lower in the exposed benzene workers, suggesting either that the control values were abnormally high or that the damaged lymphocytes may have been eliminated in the exposed workers.

As a follow-up, 48-hour cultured interphase lymphocytes from 10 individuals spanning 4 different low-exposure categories (0, ≤ 1, ≤ 5, and > 5 ppm) collected during phase 2 of the study were scored using the tandem probes for chromosome 1. A significant exposure-related increase in breakage in the 1cen-1q12 region as well as hyperdiploidy involving chromosome 1 were seen in the exposed workers (Appendix B, Table B.11). These results indicate that the tandem 1 interphase assay may be capable of detecting breaks in workers with lower-level benzene exposure. This would be consistent with an earlier relatively small study of benzene-exposed Estonian workers (Marcon et al 1997). However, the inability of this approach to detect damage in the more highly exposed workers is problematic and may indicate a serious limitation of this approach for monitoring benzene-exposed workers, particularly when the exposure levels are poorly characterized.

As indicated in the Introduction, interphase FISH can be a useful technique for detecting chromosomal changes in cells treated in vitro or those occurring in exposed human populations. This is particularly true when the effects are strong or when damage preferentially affects the regions targeted by the probes. When cells are not dividing (such as for G_0 lymphocytes) or are terminally differentiated (granulocytes), interphase analysis may be the only practical means of obtaining cytogenetic information. When the induced effects are relatively weak and do not show a preference for the targeted areas, as apparently was the case in this study, the interphase FISH approach is relatively insensitive. However, the results in our interphase FISH studies were consistent with those in our metaphase studies. Essentially no increase in hyperdiploid cells was seen in either the conventional metaphase cell or FISH analyses of the exposed workers. An increase in chromosomal loss was seen among the exposed workers in the metaphase analyses. This loss was for all chromosomes combined, and one would not expect such a loss to be detected using FISH with only one or two chromosome-specific probes. In addition, loss of chromosomes is inefficiently detected using interphase FISH. (For further discussion of this and other limitations of cytogenetic analyses using interphase FISH, see Eastmond et al 1995.) The modest increases in aneuploidy found in this study are also consistent with the results in recent studies of benzene in animals (Eastmond et al 2001). Similarly, the approximately 2-fold increases in breakage in the conventional metaphase analyses represented damage occurring throughout the genome. It would be unlikely that FISH targeting one small chromosomal region (1cen-1q12) would be capable of detecting such an increase in damage unless that region was preferentially targeted.

Although it is conceivable that differences in scoring account for some of the differences between results with this group of benzene-exposed workers and the previously published UCB/NCI FISH results, we believe this to be unlikely. To assess the reliability of our scoring, positive and negative control slides consisting of cultures treated with 0 and 2 Gy of radiation or 0 and 10 µM diethylstilbestrol were coded and scored in a blinded fashion along with the slides from phase 1 and phase 2 of the study (Appendix B, Table B.9). As expected, strong increases in structural aberrations and modest increases in aneuploid cells were seen in the irradiated cells as compared with the controls. Very strong increases in hyperdiploid cells were also seen in the diethylstilbestrol-treated cells. This established that the scoring and methods were reliable. We believe that the results indicate that the FISH assays for labeling breakage and aneuploidy in chromosome 1 and for aneuploidy affecting chromosome 7 were not sufficiently sensitive to detect the cytogenetic changes in workers with high-level benzene exposures monitored in phase 1. For comparison with the previous FISH studies of Chinese workers, our data were also analyzed in 2 subgroups divided by exposure level of 31 ppm (not shown). Again, no significant differences were detected between the unexposed group and the group exposed to benzene concentrations greater than 31 ppm. Further analysis indicated that

the median benzene exposure level in the groups with benzene exposure above 31 ppm was extremely high (91.9 ppm) in the UCB/NCI study (Rothman et al 1996) and high but substantially lower in phase 1 of our study (39.2 ppm). It is therefore possible that the large difference in benzene exposure levels between the 2 studies may account in part for the differing cytogenetic FISH results.

Owing to the negative results observed using the different FISH assays, structural and numerical aberrations for samples collected during both phases 1 and 2 also were determined using conventional cytogenetic techniques. According to the 4-week mean benzene exposure levels, the frequencies of the grouped categories of total chromatid aberrations, total chromosomal aberrations, and total aberrations (all without gaps) were significantly higher among exposed workers than among unexposed subjects (Table 27). However, the increased frequencies were not perfectly dependent on benzene exposure. All grouped chromosomal aberration outcomes were unexpectedly elevated in the low exposure group (at or below 5 ppm). In particular, the aberration frequencies in subjects exposed to benzene at or below 0.5 ppm not only were significantly higher than in unexposed subjects but also were similar to the values in the highest exposure group, > 30 ppm (Tables 27 and 29). Further analysis indicated that the median exposure duration for the group exposed to $\leq$ 0.5 ppm was 14 years while that for the > 30 ppm group was only 3.25 years.

It is possible that the observed aberrations in the workers with lower exposures were due to cumulative damage occurring over a period longer than 4 weeks. In the group with $\leq$ 0.5 ppm benzene exposure, the median level of exposure intensity was 2.7 ppm, which is relatively low compared with 39.1 ppm in the > 30 ppm group. These findings suggest that exposures higher than the median of 2.7 ppm occurring during an earlier exposure period may have contributed to the observed increase in chromosomal aberrations.

Indeed, a further analysis using cumulative exposure data revealed that the grouped chromosomal aberrations had a much better exposure-response curve (Table 30). In particular, the consistent duration-response relation (Table 31) compared with the 4-week mean exposure-response relation indicated that at the observed exposure levels, the duration of exposure was more critical and predictive of chromosomal aberrations than the currently monitored level of benzene exposure. However, among the types of structural aberration seen, chromatid-type aberrations are formed during culture as the stimulated lymphocytes replicate their DNA and prepare for mitosis (ie, during S or G_2 phase). Formation of these aberrations would usually be attributed to relatively recent exposures, although adducts and other alterations persisting for extended periods in long-lived lymphocytes could also result in these types of unstable aberrations. In addition, the observed correlations between chromosomal aberrations and albumin adducts, urinary metabolites (Table 34), and metabolite residuals (Table 35) would indicate a relation between relatively recent exposures and chromosomal aberrations.

CONCLUSIONS

The personal exposures of subjects participating in this study were fully characterized. Their observed current-day benzene exposure levels ranged from 0.06 to 122 ppm, with a median of 3.2 ppm. The median 4-week mean benzene exposure was 3.8 ppm, and the median lifetime cumulative benzene exposure was 51.1 ppm-years. The median duration of exposure to benzene was 7 years, and the median intensity of benzene exposure was 5.8 ppm/year. Compared with benzene exposure levels, current-day toluene levels were relatively high, with a median of 12.6 ppm (mean, 26.3 ppm). The current-day levels of xylene were low, with a median of 0.30 ppm (mean, 0.40 ppm).

Even though values for RBCs, WBCs, and neutrophils remained within normal ranges, significant decreases were observed in exposed subjects. In contrast, the decrease in lymphocytes associated with benzene exposure was small and only weakly significant. The depression of multiple blood cell types observed in this study indicated effects on the pluripotent stem cells, consistent with the results of other studies.

S-PMA in particular and also *t,t*-MA were the most sensitive and valid biomarkers in urine for subjects exposed to benzene at or below 1 ppm. The urinary metabolites HQ, CAT, and phenol were not reliable biomarkers for benzene exposure at or below 5 ppm owing to their high background levels from sources other than benzene exposure.

Both BO–Alb adducts and 1,4-BQ–Alb adducts were formed from benzene in an exposure-dependent manner. However, because significant background levels of these adducts were found in unexposed subjects, it is uncertain how useful they will be as biomarkers of low-level benzene exposure. Given the utility of albumin adducts as relatively long-term biomarkers, this is an important subject for future research.

The present study demonstrated that benzene exposures of only approximately 0.5 ppm could still induce some cytogenetic changes detected by traditional chromosomal aberration assays (Table 29). However, the FISH assays used in this study failed to detect any benzene-related cytogenetic changes in chromosomes 1 and 7.

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REFERENCES

Aksoy M. 1988a. Benzene carcinogenicity. In: Benzene Carcinogenicity (M Aksoy, ed), pp 113–144. CRC Press, Boca Raton FL.

Aksoy M. 1988b. Benzene hematotoxicity. In: Benzene Carcinogenicity (M Aksoy, ed), pp 59–104. CRC Press, Boca Raton FL.

Aksoy M, Dincol K, Akgun T, Erdem S, Dincol G. 1971. Hematological effects of chronic benzene poisoning in 217 workers. Br J Ind Med 28:296–302.

Aksoy M, Ozeris S, Sabuncu H, Yanardag R. 1987. Exposure to benzene in Turkey between 1983 and 1985: A hematologic study on 231 workers. Br J Ind Med 44:785–787.

Andrews LS, Lee EW, Witmer CM, Kocsis JJ, Snyder R. 1977. Effects of toluene on the metabolism, disposition and hemopoietic toxicity of [3H]benzene. Biochem Pharmacol 26:293–300.

Baselt RC. 1980. Biological Monitoring Methods for Industrial Chemicals, pp 37–42. California Biomedical Publication, Davis CA.

Bechtold WE, Henderson RF. 1993. Biomarkers of human exposure to benzene. J Toxicol Environ Health 40:366–386.

Bechtold WE, Lucier G, Birnbaum LS, Yin SN, Li GL, Henderson RF. 1991. Muconic acid determinations in urine as a biological exposure index for workers occupationally exposed to benzene. Am Ind Hyg Assoc J 52:473–478.

Bechtold WE, Sun JD, Birnbaum LS, Yin S-N, Li G-L, Kasicki S, Lucier G, Henderson RF. 1992a. S-phenylcysteine formation in hemoglobin as a biological exposure index to benzene. Arch Toxicol 66:303–309.

Bechtold WE, Willis JK, Sun JD, Griffith WC, Reddy TV. 1992b. Biological markers of exposure to benzene: S-phenylcysteine in albumin. Carcinogenesis 13:1217–1220.

Bogadi-Sare A, Turk R, Zavalic M. 1995. Medical surveillance studies of workers exposed to low level benzene. Arh Hig Rada Toksikol 46:391–398.

Bonassi S, Abbondandolo A, Camurri L, Dal Pra L, De Ferrari M, Degrassi F, Lamberti AF, Lando C, Padovani P, Sbrana I, Vecchio D, Puntoni R. 1995. Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Cancer Genet Cytogenet 79:133–135.

Boogaard PJ, van Sittert NJ. 1995. Biological monitoring of exposure to benzene: A comparison between S-phenylmercapturic acid, *trans*,*trans*-muconic acid, and phenol. Occup Environ Med 52(9):611–620.

Boogaard PJ, van Sittert NJ. 1996. Suitability of S-phenyl mercapturic acid and *trans-trans*-muconic acid as biomarkers for exposure to low concentrations of benzene. Environ Health Perspect 104 (Suppl 6):1151–1157.

Brandeau MT, Ducos P, Gaudin R, Morel G, Bonnet P, de Ceaurriz J. 1992. Evaluation of the interaction of benzene and toluene on the urinary excretion of t,t-muconic acid in rats. Toxicol Lett 61(2–3):311–316.

Carrano AV, Natarajan AT. 1988. Considerations for population monitoring using cytogenetic techniques. Mutat Res 204:379–406.

CONCAWE. 1996. Scientific basis for an air quality standard on benzene. Conservation of Clean Air and Water in Western Europe, Brussels, 96/63.

Conforti-Froes N, El-Zein R, Abdel-Rahman SZ, Zwischenberger JB, Au WW. 1997. Predisposing genes and increased chromosome aberrations in lung cancer cigarette smokers. Mutat Res 3799:53–59.

Conover WJ, Iman R. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. Am Stat 35:124–129.

Cooke HJ, Hindley J. 1979. Cloning of human satellite III DNA: Different components are on different chromosomes. Nucleic Acids Res 6:3177–3197.

Ding X, Li Y, Ding Y, Yang H. 1983. Chromosome changes in patients with chronic benzene poisoning. Chin Med J 96:681–685.

Dor F, Dab W, Empereur-Bissonnet P, Zmirou D. 1999 Validity of biomarkers in environmental health studies: The case of PAHs and benzene. Crit Rev Toxicol 29:129–168.

Dosemeci M, Li G-L, Hayes RB, Yin S-N, Linet M, Chow WH, Wang Y-Z, Jiang Z-L, Dai T-R, Zhang W-U, Chao X-J, Ye P-Z, Kou Q-R, Fan Y-H, Zhang X-C, Lin X-F, Meng J-F, Zho J-S, Wacholder S, Kneiler R, Blot WJ. 1994. Cohort study among workers exposed to benzene in China: II. Exposure assessment. Am J Ind Med 26:401–411.

Ducos P, Gaudin R, Bel J, Maire C, Francin JM, Robert A, Wild P. 1992. *trans,trans*-Muconic acid, a reliable biological indicator for the detection of individual benzene exposure down to the ppm level. Int Arch Occup Environ Health 64:309–313.

Ducos P, Gaudin R, Robert A, Francis JM, Maire C. 1990. Improvement in HPLC analysis of urine *t,t*-muconic acid, a promising substitute for phenol in the assessment of benzene exposure. Int Arch Occup Environ Health 62:529–534.

Eastmond DA. 1993. Induction of micronuclei and aneuploidy by the quinone-forming agents benzene and o-phenylphenol. Toxicol Lett 67:105–118.

Eastmond DA, Pinkel D. 1990. Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence in situ hybridization with chromosome-specific DNA probes. Mutat Res 234:303–318.

Eastmond DA, Rupa DS. 1995. Fluorescence in situ hybridization: Application to environmental mutagenesis. In: Environmental Mutagenesis (DH Phillips, S Venitt, eds), pp 261–290. Bios Scientific Publications, Oxford, UK.

Eastmond DA, Rupa DS, Hasegawa LS. 1994. Detection of hyperdiploidy and chromosome breakage in interphase human lymphocytes following exposure to the benzene metabolite hydroquinone using multicolor fluorescence in situ hybridization with DNA probes. Mutat Res 322:9–20.

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. HEI Research Report 103. Health Effects Institute, Cambridge MA.

Eastmond DA, Schuler M, Rupa DS. 1995. Advantages and limitations of using fluorescence in situ hybridization for the detection of aneuploidy in interphase human cells. Mutat Res 348:153–162.

Ehrenberg L, Hiesche KD, Osterman-Golkar S, Wennberg I. 1974. Evaluation of genetic risks of alkylating agents: Tissue doses in the mouse from air contaminated with ethylene oxide. Mutat Res 24:83–103.

Eller PM. 1984. Method 1501. In: NIOSH manual of analytical methods (3rd ed). Publ 84-100. US Department of Health and Human Services, Cincinnati OH.

Fleiss JL. 1986. The Design and Analysis of Clinical Experiments. John Wiley & Sons, New York NY.

Forni A. 1979. Chromosome changes and benzene exposure: A review. Rev Environ Health 3:5–7.

Forni A, Cappellini A, Pacifico E, Vigliani EC. 1971. Chromosome changes and their evolution in subjects with past exposure to benzene. Arch Environ Health 23:385–391.

Gad-El Karim MM, Ramanujam VM, Legator MS. 1985. *trans,trans*-Muconic acid, an open-chain urinary metabolite of benzene in mice: Quantification by high-pressure liquid chromatography. Xenobiotica 15:211–220.

Ghittori S, Imbriani M, Maestri L, Capodaglio E, Cavalleri A. 1999. Determination of S-phenylmercapturic acid in urine as an indicator of exposure to benzene. Toxicol Lett 108:329–334.

Ghittori S, Maestri L, Fiorentino ML, Imbriani M. 1995. Evaluation of occupational exposure to benzene by urinalysis. Int Arch Occup Environ. Health 67:195–200.

Ghittori S, Maestri L, Rolandi L, Lodola L, Fiorentino ML, Imbriani M. 1996. The determination of *trans,trans*-muconic acid in urine as an indicator of occupational exposure to benzene. Appl Occup Environ Hyg 11:187–191.

Goldstein BD. 1977. Hematotoxicity in humans. J Toxicol Environ Health 2(Suppl):69–105.

Goldstein BD. 1988. Benzene toxicity. Occup Med 3:541–554.

Goldwater LJ. 1941. Disturbances in the blood following exposure to benzol. J Lab Clin Med 26:957–973.

Gray JW, Pinkel D. 1992. Molecular cytogenetics in human cancer diagnosis. Cancer 69(Suppl):1536–1542.

Hagmar L, Bonassi S, Stromberg U, Brogger A, Knudsen LE, Norppa H, Reuterwall C. 1998. Chromosomal aberrations in lymphocytes predict human cancer: A report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). Cancer Res 58:4117–4121.

Hagmar L, Brogger A, Hansteen I-L, Heim S, Hogstedt B, Knudsen L, Lambert B, Linnainmaa K, Mitelman F, Nordenson I, Reuterwall C, Salomaa S, Skerfving S, Sorsa M. 1994. Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. Cancer Res 54:2919–2922.

Haley NJ, Axelrad CM, Tilton K. 1983. Validation of self-reported smoking behavior: Biochemical analyses of cotinine and thiocyanate. Am J Public Health 73:1204–1207.

Hanway R, Cavicchioli A, Kaur B, Parsons J, Lamb JH, Buckberry LD, Farmer PB. 2000. Analysis of S-phenyl-L-cysteine in globin as marker of benzene exposure. Biomarkers 4:252–262.

Hasegawa LS, Rupa DS, Eastmond DA. 1995. A method for the rapid generation of alpha- and classical satellite probes for human chromosome 9 by polymerase chain reaction using genomic DNA and their application to detect chromosomal alterations in interphase cells. Mutagenesis 10:471–476.

Henderson RF, Sabourin PJ, Bechtold WE, Griffith WC, Medinsky MA, Birnbaum LS, Lucier GW. 1989. The effect of dose, dose rate, route of administration, and species on tissue and blood levels of benzene metabolites. Environ Health Perspect 82:9–17.

Hernberg S, Savilahti M, Ahlman K, Asp S. 1966. Prognostic aspects of benzene poisoning. Br J Ind Med 23:204–209.

Hotz P, Carbonnelle P, Haufroid V, Tschopp A, Buchet JP, Lauwerys R. 1997 Biological monitoring of vehicle mechanics and other workers exposed to low concentrations of benzene. Int Arch Occup Environ Health 70:29–40.

Ikeda M, Inoue O. 1995. Determination of phenolic metabolites and *t,t*-muconic acid in urine of benzene-exposed workers. In: Advances in Occupational Medicine and Rehabilitation Vol 1, No 2, pp 201–207. Fondazione Salvatore Maugeri Edizioni, Pavia, Italy.

Inoue O, Seiji K, Kasahara M, Nakatsuka H, Watanabe T, Yin SG, Li GL, Cai SX, Jin C, Ikeda M. 1988b. Determination of catechol and quinol in the urine of workers exposed to benzene. Br J Ind Med 45(7):487–492.

Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin S-N, Li G-L, Cai S-X, Jin C, Ikeda M. 1989a. Urinary *t,t*-muconic acid as an indicator of exposure to benzene. Brit J Ind Med 46:122–127.

Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin S-N, Li G-L, Cai S-X, Jin C, Ikeda M. 1989b. Excretion of 1,2,4-benzenetriol in the urine of workers exposed to benzene. Br J Ind Med 46:559–565.

Inoue O, Seiji K, Watanabe T, Kasahara M, Nakatsuka H, Yin SN, Li GL, Cai SX, Jin C, Ikeda M. 1988a. Mutual metabolic suppression between benzene and toluene in man. Int Arch Occup Environ Health 60:15–20.

International Agency for Research on Cancer. 1982. Benzene. In: Some Industrial Chemicals and Dyestuffs, pp 93–148. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans Vol 29. IARC, Lyon, France.

International Agency for Research on Cancer. 1997. Workshop report. In: Application of Biomarkers in Cancer Epidemiology, pp 1–18. IARC Scientific Publications 142. IARC, Lyon, France.

Johnson ES, Lucier G. 1992. Perspectives on risk assessment impact of recent reports on benzene. Am J Ind Med 21:749–757.

Khuder SA, Youngdale MC, Bisesi MS, Schaub EA. 1999. Assessment of complete blood count variations among workers exposed to low levels of benzene. J Occup Environ Med 41:821–826.

Kipen, HM, Cody RP, Goldstein BD. 1989. Use of longitudinal analysis of peripheral blood counts to validate historical reconstructions of benzene exposure. EnvironHealth Perspect 82:199–206.

Kivisto H, Pekari K, Peltonen K, Svinhufvud J, Veidebaum T, Sorsa M, Aitio A. 1997. Biological monitoring of exposure to benzene in the production of benzene and in a cokery. Sci Total Environ 199:49–63

LaMontagne AD, Christiani DC, Kelsey KT. 1993. Utility of the complete blood count in routine medical surveillance for ethylene oxide exposure. Am J Ind Med 24:191–206.

Lee BL, Ong HY, Shi CY, Ong CN. 1993. Simultaneous determination of hydroquinone, catechol and phenol in urine using high-performance liquid chromatography with fluorimetric detection. J Chromatogr 619:259–266.

Lindstrom AB, Yeowell-O'Connell K, Waidyanatha S, McDonald TA, Golding BT, Rappaport SM. 1998. Formation of hemoglobin and albumin adducts of benzene oxide in mouse, rat, and human blood. Chem Res Toxicol 11:302–310.

Liniecki J, Bajerska A, Gluszczowa M. 1971. Analiza kariologiczna limfocytow krwi obwodowej u osob z przebytym

przewleklym zatruciem benzenem. Medycyna Pracy 22:187–193.

Maestri L. 1995. Determination of hemoglobin adducts formed by benzene exposure. In: Advances in Occupational Medicine and Rehabilitation Vol 1, No 2, pp 219–229. Fondazione Salvatore Maugeri Edizioni, Pavia, Italy.

Major J, Jakab M, Kiss G, Tompa A. 1994. Chromosome aberration, sister-chromatid exchange, proliferative rate index, and serum thiocyanate concentration in smokers exposed to low-dose benzene. Environ Mol Mutagen 23:137–142.

Marcon F, Zijno A, Crebelli R, Carere A, Schuler M, Parks R, Eastmond DA. 1997. Detection of cytogenetic damages in blood cells of benzene-exposed workers by multicolor fluorescence in situ hybridization (FISH). Mutat Res 379:S141, XIV C.23.

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

Martin RH, Ernst S, Rademaker A, Barclay L, Ko E, Summers N. 1997. Chromosomal abnormalities in sperm from testicular cancer patients before and after chemotherapy. Hum Genet 99:214–218.

McDonald TA, Waidyanatha S, Rappaport SM. 1993. Measurement of adducts of benzoquinone with hemoglobin and albumin. Carcinogenesis 14(9):1927–1932.

Melikian AA, Meng M, O'Connor R, Hu P, Thompson SM. 1999a. Development of Liquid Chromatography–Electrospray Ionization–-Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans. Research Report 87. Health Effects Institute, Cambridge MA.

Melikian AA, O'Connor R, Prahalad AK, Hu P, Li H, Kagan M, Thompson S. 1999b. Determination of urinary benzene metabolites S-phenylmercapturic acid and *trans*,*trans*-muconic acid by liquid chromatography-tandem mass spectrometry. Carcinogenesis 20:719–726.

Melikian AA, Prahalad AK, Coleman S. 1992. Isolation and characterization of the two benzene-derived hemoglobin adducts in vivo in rats. Cancer Epidemiol Biomarkers, Prev. 1:307–313.

Melikian AA, Prahalad AK, Hoffmann, D. 1993. Urinary *trans,trans*-muconic acid as an indicator of exposure to benzene in cigarette smokers. Cancer Epidemiol Biomarkers Prev 2:47–51.

Melikian AA, Prahalad AK, Secker-Walker RH. 1994. Comparison of levels of urinary benzene metabolite *trans*,*trans*-muconic acid in smokers and nonsmokers, and the effects of pregnancy. Cancer Epidemiol Biomarkers Prev 3:239–244.

Ong CN, Kok PW, Lee BL, Shi CY, Ong HY, Chia KS, Lee CS, Luo XW. 1995. Evluation of biomarkers of occupational exposure to benzene. Occup Environ Med 52:528–533.

Ong CN, Kok PW, Ong HY, Shi CY, Lee BL, Phoon WH, Tan KT. 1996. Biomarkers of exposure to low concentrations of benzene: A field assessment. Occup Environ Med 53:328–333.

Ong CN, Lee BL. 1994. Determination of benzene and its metabolites: Application in biological monitoring of environmental and occupational exposure to benzene. J Chromatogr B Biomed Appl 660:1–22.

Osterman-Golkar S, Christakopoulos A, Segerback D, Hallstrom I. 1976. Evaluation of genetic risks of alkylating agents II: Hemoglobin as a dose monitor. Mutat Res 34:1–10.

Pekari K. 1995. Biological monitoring of occupational exposure to low levels of benzene. In: Advances in Occupational Medicine and Rehabilitation Vol 1, No 2, pp 145–154. Fondazione Salvatore Maugeri Edizioni, Pavia, Italy.

Pezzagno G, Maestri L. 1997. The specifics of *trans,trans*-muconic acid as a biological indicator for low levels of environmental benzene. Indoors Built Environ 6:12–18.

Pezzagno G, Maestri L, Fiorentino ML. 1999. *trans,trans*-Muconic acid, a biological indicator to low levels of environmental benzene: Some aspects of its specificity. Am J Ind Med 35(5):511–518.

Pollini G, Biscaldi GP. 1976. Persistenza delle alterazioni del cariotipo nei linfociti di soggetti benzolici a dieci anni dalla intossicazione. Med Lav 67:465–472.

Pollini G, Biscaldi GP, Robustelli Della Cuna G. 1969. [Chromosome changes of the lymphocytes discovered after five years in subjects with benzolic hemopathy.] Med Lav 60:743–758.

Popp W, Rauscher D, Muller G, Angerer J, Norpoth K. 1994. Concentrations of benzene in blood and S-phenylmercapturic acid and *t,t*-muconic acid in urine in car mechanics. Int Arch Occup Environ Health 66:1–6.

Qu QS, Melikian AA, Li G, Shore R, Chen LC, Cohen B, Yin S, Kagan M, Li H, Meng M, Jin X, Winnik W, Li Y, Mu R, Li K. 2000. Validation of biomarkers in humans exposed to benzene: I. Urine metabolites. Am J Ind Med 37:522–531.

Ramirez MJ, Surralles J, Galofre P, Creus A, Marcos R. 1997. Radioactive iodine induces clastogenic and age-dependent aneugenic effects in lymphocytes of thyroid cancer patients as revealed by interphase FISH. Mutagenesis 12:449–455.

Rappaport SM, Yeowell-O'Connell K. 1999. Protein adducts as dosimeters of human exposure to styrene, styrene-7,8-oxide and benzene. Toxicol Lett 108:117–126.

Robbins WA, Baulch JE, Moore D, Weier HU, Blakey D, Wyrobek AJ. 1995. Three-probe fluorescence in situ hybridization to assess chromosome X, Y, and 8 aneuploidy in sperm of 14 men from two healthy groups: Evidence for a paternal age effect on sperm aneuploidy. Reprod Fertil Dev 7:799–809.

Robbins WA, Vine MF, Truong KY, Everson RB. 1997. Use of fluorescence in situ hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men. Environ Mol Mutagen 30:175–183.

Rothman N, Li G, Dosemeci M, Bechtold WE, Marti GE, Wang Y, Linet M, Xi L, Lu W, Smith MT, Titenko-Holand N, Zhang L, Blot W, Yin S, Hayes R. 1996. Hematotoxicity among Chinese workers heavily exposed to benzene. Am J Ind Med 29:236–246.

Rump P. 1981. Darstellung, Charakterisierung und Trennung der Mercaptursäurea von Benzol, Totuol, Aethylbenzol und Xylolen Durch GC-MS-Analyse [dissertation], pp 20–25. University of Hamburg, Hamburg, Germany.

Rupa DS, Eastmond DA. 1997. Chromosomal alterations affecting the 1cen-1q12 region in buccal mucosal cells of betel quid chewers detected using multicolor fluorescence in situ hybridization. Carcinogenesis 18:2347–2351.

Rupa DS, Hasegawa L, Eastmond DA. 1995. Detection of chromosomal breakage in the 1 cen-1q12 region of interphase human lymphocytes using multicolor fluorescence in situ hybridization with tandem DNA probes. Cancer Res 55:640–645.

Rupa DS, Schuler M, Eastmond DA. 1997. Detection of hyperdiploidy and breakage affecting the 1cen-1q 12 region of cultured interphase human lymphocytes treated with various genotoxic agents. Environ Mol Mutagen 29:161–167.

Ruppert T, Scherer G, Tricker AR, Adlkofer F. 1997. *trans,trans*-Muconic acid as a biomarker of non-occupational environmental exposure to benzene. Int Arch Occup Environ Health 69:247–251.

Ruppert T, Scherer G, Tricker AR, Rauscher D, Adlkofer F. 1995. Determination of urinary *trans,trans*-muconic acid by gas chromatography–mass spectrometry. J Chromatogr B Biomed Sci Appl 666:71–76.

Sabourin PJ, Sun JD, Birnbaum LS, Lucier G, Henderson RF. 1989. Effect of repeated benzene inhalation exposures on subsequent metabolism of benzene. Exp Pathol 37:155–157.

Sarto F, Cominato I, Pinton AM, Brovedani PG, Merler E, Peruzzi M, Bianchi V, Levis AG. 1984. A cytogenetic study on workers exposed to low concentrations of benzene. Carcinogenesis 5:827–832

SAS. 2000. SAS Statistical System. SAS Institute, Cary NC.

Sato A, Nakajima T. 1979. Dose-dependent metabolic interaction between benzene and toluene in vivo and in vitro. Toxicol Appl Pharmacol 48:249–256.

Schuler M, Hasegawa L, Parks R, Metzler M, Eastmond DA. 1998. Dose-response studies of the induction of hyperdiploidy and polyploidy by diethylstilbestrol and 17beta-estradiol in cultured human lymphocytes using multicolor fluorescence in situ hybridization. Environ Mol Mutagen 31:263–273.

Smith MT, Zhang L, Wang Y, Hayes RB, Li G, Wiemels J, Dosemeci M, Titenko-Holland N, Xi L, Kolachana P, Yin S, Rothman N. 1998. Increased translocations and aneusomy in chromosomes 8 and 21 among workers exposed to benzene. Cancer Res 58:2176–2181.

Sorsa M, Wilbourn J, Vainio H. 1992. Human cytogenetic damages as a predictor of cancer risk. In: Mechanisms of Carcinogenesis in Risk Identification (H Vainio, PN Magee, DB McGregor, AJ McMichael, eds), pp 543–554. IARC Scientific Publications 116. International Agency for Research on Cancer, Lyon, France.

SPSS. 1994. Base system user's guide; Syntax reference guide; Advanced statistics. In: SPSS for Windows, Release 6.1 (Norusis M, ed). SPSS, Chicago IL.

Stommel P, Muller G, Stucker W, Verkoyen C, Schobel S, Norpoth K. 1989. Determination of S-phenylmercapturic acid in the urine: an improvement of the biological monitoring of benzene exposure. Carcinogenesis 10:279–282.

Surralles J, Autio k, Nylund L, Jarventaus H, Norppa H, Veidebaum T, Sorsa M, Peltonen K. 1997a. Molecular cytogenetic analysis of buccal cells and lymphocytes from benzene-exposed workers. Carcinogenesis 18:817–823.

Surralles J, Darroudi F, Natarajan AT. 1997b. Low level of DNA repair in human chromosome 1 heterochromatin. Genes Chromosom Cancer 20:173–184.

Tagarro I, Fernandez-Peralta AM, Gonzalez-Aguilera JJ. 1994. Chromosomal localization of human satellites 2 and 3 by a FISH method using oligonucleuotides as probes. Hum Genet 93:383–388.

Tompa A, Major J, Jakab M. 1994. Monitoring of benzene-exposed workers for genotoxic effects of benzene: Improved-working-condition-related decrease in the frequencies of chromosome aberrations in peripheral blood lymphocytes. Mutat Res 304:159–165.

Trask B, Pinkel D. 1990. Fluorescence in situ hybridization with DNA probes. Methods Cell Biol 33:383–400.

Troester MA, Lindstrom AB, Kupper LL, Waidyanatha S, Rappaport SM. 2000. Stability of hemoglobin and albumin adducts of benzene oxide and 1,4-benzoquinone after administration of benzene to F344 rats. Toxicol Sci 54:88–94.

Tucker JD, Eastmond DA, Littlefield LG. 1997. Cytogenetic end-points as biological dosimeters and predictors of risk in epidemiological studies. In: Application of Biomarkers in Cancer Epidemiology, pp 185–200. IARC Scientific Publications 142. International Agency for Research on Cancer, Lyon, France.

Turkel B, Egeli U. 1994. Analysis of chromosomal aberrations in shoe workers exposed long term to benzene. Occup Environ Med 51:50–53.

van Sittert NJ, Boogaard PJ, Beulink GDJ. 1993. Application of urinary S-phenylmercapturic acid test as a biomarker for low levels of exposure to benzene in industry. Brit J Ind Med 50:460–469.

Vogel E, Gunther H. 1967. Benzeoxide-oxepin valence tautomerism. Agnew Chem Int Edit Engl 6:385–401.

Waidyanatha S, Yeowell-O'Connell K, Rappaport SM. 1998. A new assay for albumin and hemoglobin adducts of 1,2- and 1,4- benzoquinones. Chem Biol Interact 115(2):117–139.

Wallace L. 1996. Environmental exposure to benzene: An update. Environ Health Perspect 104(Suppl 6):1129–1136.

Ward CO, Kuna RA, Snyder NK, Alsaker RD, Coate WB, Craig PH. 1985. Subchronic inhalation toxicity of benzene in rats and mice. Am J Ind Med 7:457–473.

Ward E, Hornung R, Morris J, Rinsky R, Wild D, Halperin W, Guthrie W. 1996. Risk of low red or white blood cell count related to estimated benzene exposure in a rubber-worker cohort (1940–1975). Am J Ind Med 29:247–257.

World Health Organization. 1993. Biomarkers and Risk Assessment: Concepts and Principles. WHO Environmental Health Criteria 155. World Health Organization, Geneva, Switzerland.

Witz G, Maniara W, Mylavarapu V, Goldstein BD. 1990. Comparative metabolism of benzene and trans,trans-muconaldehyde to trans,trans-muconic acid in DBA/2N and C57BL/6 mice. Biochem Pharmacol 40(6):1275–1280.

Witz G, Zhang Z, Goldstein BD. 1996. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. Environ Health Perspect 104(Suppl 6):1195–1199.

Yardley-Jones A, Andweson D, Park DV. 1991. The toxicity of benzene and its metabolism and molecular pathology in human risk assessment. Br J Ind Med 48:437–444.

Yeowell-O'Connell K, Rothman N, Smith MT, Hayes RB, Li G, Waidyanatha S, Dosemeci M, Zhang L, Yin S, Titenko-Holland N, Rappaport SM. 1998. Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. Carcinogenesis 19:1565–1571.

Yu R, Weisel CP. 1996. Measurement of the urinary benzene metabolite trans,trans-muconic acid from benzene exposure in humans. J Toxicol Environ Health 48:453–477.

Zar JH. 1984. Biostatistical Analysis (2nd ed). Prentice-Hall, Englewood Cliffs NJ.

Zhang L, Rothman N, Wang Y, Hayes RB, Bechtold W, Venkatesh P, Yin S, Wang Y, Dosemeci M, Li G, Lu W, Smith MT. 1996. Interphase cytogenetics of workers exposed to benzene. Environ Health Perspect 104(Suppl 6):1325–1329.

Zhang L, Rothman N, Wang Y, Hayes RB, Li G, Dosemeci M, Yin S, Kolachana P, Titenko-Holland N, Smith MT. 1998. Increased aneusomy and long arm deletion of chromosomes 5 and 7 in the lymphocytes of Chinese workers exposed to benzene. Carcinogenesis 19:1955–1961.

APPENDICES A and B AVAILABLE ON REQUEST

The following appendices may be downloaded as PDF files from *www.healtheffects.org*. Hard copies may be requested by contacting the Health Effects Institute and providing the author name, full title and number of the report, and titles of appendices you request.

Appendix A. Analyses of the Combined Data for Year 1 and Year 2

Appendix B. Additional Statistical Analyses

APPENDIX C. HEI Quality Assurance Report

The conduct of this study was subject to periodic, independent audits by a team from Arthur D Little. This team consisted of auditors with experience in toxicology, industrial hygiene, epidemiology, and analytical chemistry. The audits included in-process monitoring of study activities for conformance to the study protocol and examination of records and supporting data. The dates of each audit are listed in the table below with the phase of the study examined:

Date	Phase of Study Audited
4/28–5/12/1997	Site visit to: National Institute of Occupational Medicine of the Chinese Academy of Preventive Medicine, Beijing, China
	In-process monitoring for conformance to SOPs
10/28–29/1997	Site visits to: American Health Foundation, Valhalla NY
	New York University, Tuxedo NY
	In-process monitoring for conformance to SOPs
4/16–20/1998	Site visits to: Chinese Academy of Preventive Medicine, Beijing, China
	Tianjin Institute of Industrial Hygiene & Occupational Disease, Tianjin, China
	Tianjin South China Leesheng Sporting Goods Co Ltd, Tianjin, China
	In-process monitoring for conformance to SOPs
10/25/2000	Site visit to: New York University, Tuxedo NY
	Draft final report audit

Written reports for each inspection were provided to the Director of Research of the Health Effects Institute, who transmitted these findings to the Principal Investigator. These quality assurance audits demonstrated that this study was conducted by a well-coordinated, experienced team of professionals in accordance with the study protocol and standard operating procedures. Exceptions noted during the course of the audits were not of a nature to significantly compromise the draft final report, or the data contained therein. The report appears to be an accurate representation of the study.

Martin Anderson CiH, CSP
Manager

ABOUT THE AUTHORS

Qingshan Qu is an assistant professor of environmental medicine, NYU School of Medicine. Dr Qu received his MD in 1969 from Beijing Medical College, China, and completed his residency in occupational medicine at the Third Teaching Hospital of Beijing Medical College in 1973. His research interests include biomarker application and risk assessment in humans, as well as evaluation of the roles that metabolic gene polymorphisms play in human diseases with environmental origins.

Roy Shore has received both a PhD in psychology (Syracuse University, 1967) and a DrPH in epidemiology (Columbia University, 1982). He is a tenured professor of environmental medicine, NYU Medical School, and director of the Epidemiology Division. He is also director of the Epidemiology and Prevention Program in the NYU Cancer Center. His research interests include environmental and radiation epidemiology, and cancer susceptibility in relation to variation in metabolic and DNA repair genes.

Guilan Li is a professor of toxicology at the Institute of Occupational Medicine, Center for Disease Control and Prevention, Beijing, China. She received her PhD degree in 1965 from Jilin University, China. She serves as director of the Toxicology Department and has been collaborating with NCI for many years on benzene studies conducted in China. Her research interests focus on molecular epidemiology and inhalation toxicology.

Lun Chi Chen received his PhD in environmental health sciences from NYU School of Medicine in 1983. He is an associate professor of environmental medicine, NYU School of Medicine, and serves as director of the NYU NIEHS Center's Information Services facility. Dr Chen's research interests include health effects of air pollution, and cellular and molecular mechanisms of lung injury.

Beverly S Cohen is a professor of environmental medicine, NYU School of Medicine. She serves as director of the NYU School of Medicine NIEHS Center's Shared Resource in Inhalation and Exposure Assessment and of the Exposure, Dosimetry, and Modelling Research Core of the EPA PM_{10} Center. Dr Cohen received her PhD in environmental health sciences from NYU in 1979, her MS in radiological physics from Cornell University Graduate School of Medical Sciences in 1961, and her BS in physics from Bryn Mawr College in 1953. Her research interests include measurement of personal exposures to airborne toxicants, dosimetry of inhaled pollutant gases and aerosols, airborne radioactivity, and development of biomarkers for assessment of personal exposure.

Assieh A Melikian is head of the Section of Carcinogen Biomarkers at the Institute for Cancer Prevention, formerly American Health Foundation. She received her MS in chemical engineering from Tehran University, Iran, and her PhD in bio-organic chemistry from NYU. Her research interests are mechanisms of chemical carcinogenesis, tobacco-related carcinogenesis, and transitional epidemiology studies.

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 a fluorescent in situ hybridization to rapidly assess chromosomal damage caused by environmental and occupational chemicals in human populations.

SM Rappaport received his PhD in environmental sciences and engineering from the University of North Carolina in 1973. He has been a professor of occupational health at the University of North Carolina since 1990, prior to which he was a professor at the University of California, Berkeley, for 14 years. Since 1974, he has been actively engaged in various areas of research involving both environmental and biological monitoring. His current research focuses upon human dosimetry of various genotoxic chemicals, including benzene and styrene, and upon elucidating mechanisms of toxicity of these substances.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Melikian AA, Qu QS, Shore R, Li G, Li H, Jin X, Cohen B, Chen LC, Li Y, Yin S, Mu R, Zhang X, Wang Y. 2002. Personal exposure to different levels of benzene and its relationships to the urinary metabolites *S*-phenylmercapturic acid and *trans,trans*-muconic acid. J Chromatogr B 778:200–221.

Qu QS, Shore R, Li G, Jin X, Chen LC, Cohen B, Melikian AA, Eastmond D, Rappaport SM, Yin S, Li H, Waidyanatha, S, Li Y, Mu R, Zhang X, Li K. 2002. Hematological changes among Chinese workers with a broad range of benzene exposures. Am J Ind Med 42:275–285.

Rappaport SM, Waidyanatha S, Qu QS, Shore R, Jin X, Cogen B, Chen LC, Melikian AA, Li G, Yin S, Yan H, Xu B, Mu R, Li Y, Zhang X, Li K. 2002. Albumin adducts of benzene oxide and 1,4-benzoquinone as measures of human benzene metabolism. Cancer Res 62:1330–1337.

Qu QS, Melikian AA, Li G, Shore R, Chen LC, Cohen B, Yin S, Kagan M, Li H, Meng M, Jin X, Winnik W, Li Y, Mu R, Li K. 2000. Validation of biomarkers in humans exposed to benzene: I. Urine metabolites. Am J Indust Med 37:522–531.

ABBREVIATIONS AND OTHER TERMS

1,4-BQ–Alb	adduct of 1,4-benzoquinone and albumin
1,2-BQ–Alb	adduct of 1,2-benzoquinone and albumin
ANOVA	analysis of variance
BO	benzene oxide
BO–Alb	adduct of benzene oxide and albumin
BQ	benzoquinone (1,2-BQ; 1,4-BQ)
BT	benzene triol
CAT	catechol
CI	confidence interval
Cu_2O	cuprous oxide

$Cu(NO_3)_2$	copper nitrate
$CuSO_4$	copper sulfate
CV	coefficient of variation
CYP2E1	cytochrome P450 2E1
CYP450	cytochrome P450
DAPI	4′,6-diamidino-2-phenylindole
df	degrees of freedom
EPA	Environmental Protection Agency (US)
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
GC	gas chromatography
GC-MS	gas chromatography–mass spectrometry
H_2SO_4	sulfuric acid
HCl	hydrochloric acid
HPLC	high-pressure liquid chromatography
HQ	hydroquinone
HQ-*S*-TFA	*O,O′,S-tris*-trifluoroacetyl-hydroquinone
IARC	International Agency for Research on Cancer
IgG	immunoglobulin G
KCl	potassium chloride
LC-ES-MS/MS	liquid chromatography–electrospray ionization–tandem mass spectrometry
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
MeOH	methanol
$NaNO_2$	sodium nitrite
NCI	National Cancer Institute (US)
NIEHS	National Institute of Environmental Health Sciences
NIST	National Institute of Standards and Technology (US)
NYU	New York University
OSHA	Occupational Safety and Health Administration (US)
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
ppm-years	estimated lifetime cumulative occupational exposure
PTTA	phenyltrifluorothioacetate
Q	quadrupole
r	bivariate correlation coefficient
R	multivariate correlation coefficient
RBC	red blood cell
RFA	Request for Applications
RFQ	Request for Qualifications
S-PMA	*S*-phenylmercapturic acid
SSC	standard saline citrate
t,t-MA	*trans,trans*-muconic acid
UCB	University of California at Berkeley
WBC	white blood cell

COMMENTARY
Health Review Committee

INTRODUCTION

Exposure to benzene is widespread because it is a component of gasoline and is also used extensively as an industrial solvent. Ambient air concentrations are typically 1 to 10 ppb, equivalent to 3.2 to 32 $\mu g/m^3$ (Wallace et al 1996), but occupational exposures may be 1000-fold (1 to 5 ppm) or even higher. In humans and other species, chronic exposure to high concentrations of benzene induces a spectrum of conditions including cancer. On the basis of findings from occupational epidemiologic studies, the US Environmental Protection Agency (EPA*) has classified benzene as a known human carcinogen (EPA 1998). In addition, the US Occupational Safety and Health Administration (OSHA), which regulates benzene levels at work sites, has set a current permissible exposure level of 1.0 ppm in air as an 8-hour time-weighted average (OSHA 1987).

Several critical questions about the toxic effects of benzene remain unanswered, however: in particular, the mechanisms by which benzene induces toxic effects and the types of effects that occur in humans exposed to ambient levels of benzene. These issues are difficult to address. Benzene metabolism is complex: metabolites are generated by different pathways, some can be derived from processes other than the metabolism of benzene, and different metabolites are generated at different benzene exposure levels. In addition, accurately and sensitively measuring metabolites of benzene, particularly those produced at low levels, has proved challenging.

In the 1990s, Health Effects Institute initiated a research program designed to improve understanding of the effects from exposure to toxic air pollutants at ambient levels. As one part of this program, HEI's Request for Applications (RFA) 93-1 supported studies to develop sensitive assays for biomarkers of recent and longer-term benzene exposure and benzene effect. (These studies are described in the Scientific Background section.) HEI also funded parallel studies addressing the mechanism and toxicity of 1,3-butadiene.

Another part of HEI's research program was to validate the biomarkers characterized in the studies funded by RFA 93-1. Request for Qualifications (RFQ) 95-3, "Transitional Epidemiology Studies for Benzene or 1,3-Butadiene Biomarkers," solicited applications from investigators with access to human populations exposed to benzene or butadiene that would be suitable for such studies. More details of the RFQ 95-3 selection procedure are given in the Commentary to HEI Research Report 116, a transitional epidemiologic study of 1,3-butadiene biomarkers (Albertini et al 2003).

In response to RFQ 95-3, Dr Qingshan Qu, of New York University School of Medicine, and his colleagues proposed a 2-part study of workers occupationally exposed to benzene in China. In phase 1, Qu would collaborate with the HEI-funded investigators who had identified possible benzene biomarkers under RFA 93-1. They would evaluate the applicability of each biomarker to the analysis of blood and urine samples from a small number of benzene-exposed and unexposed workers. In phase 2, they would evaluate relations between benzene exposures and levels of the validated biomarkers in a large panel of workers. The investigators would focus on obtaining samples from workers exposed at the low end of the occupational exposure range. The HEI Research Committee believed that Qu and colleagues' 2-stage approach would provide important information about possible benzene biomarkers in occupationally exposed workers and recommended their proposed study for funding.[†]

At the end of the study, Qu's draft Investigators' Report underwent external peer review under direction of the HEI Health Review Committee, which discussed the report and the reviewers' critiques and prepared the 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 into scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

BENZENE METABOLISM AND MECHANISM OF TOXICITY

In humans and other species, exposure to benzene can be toxic to bone marrow and the blood cells it produces. Depending on the level and duration of exposure, the benzene-induced spectrum of human conditions includes

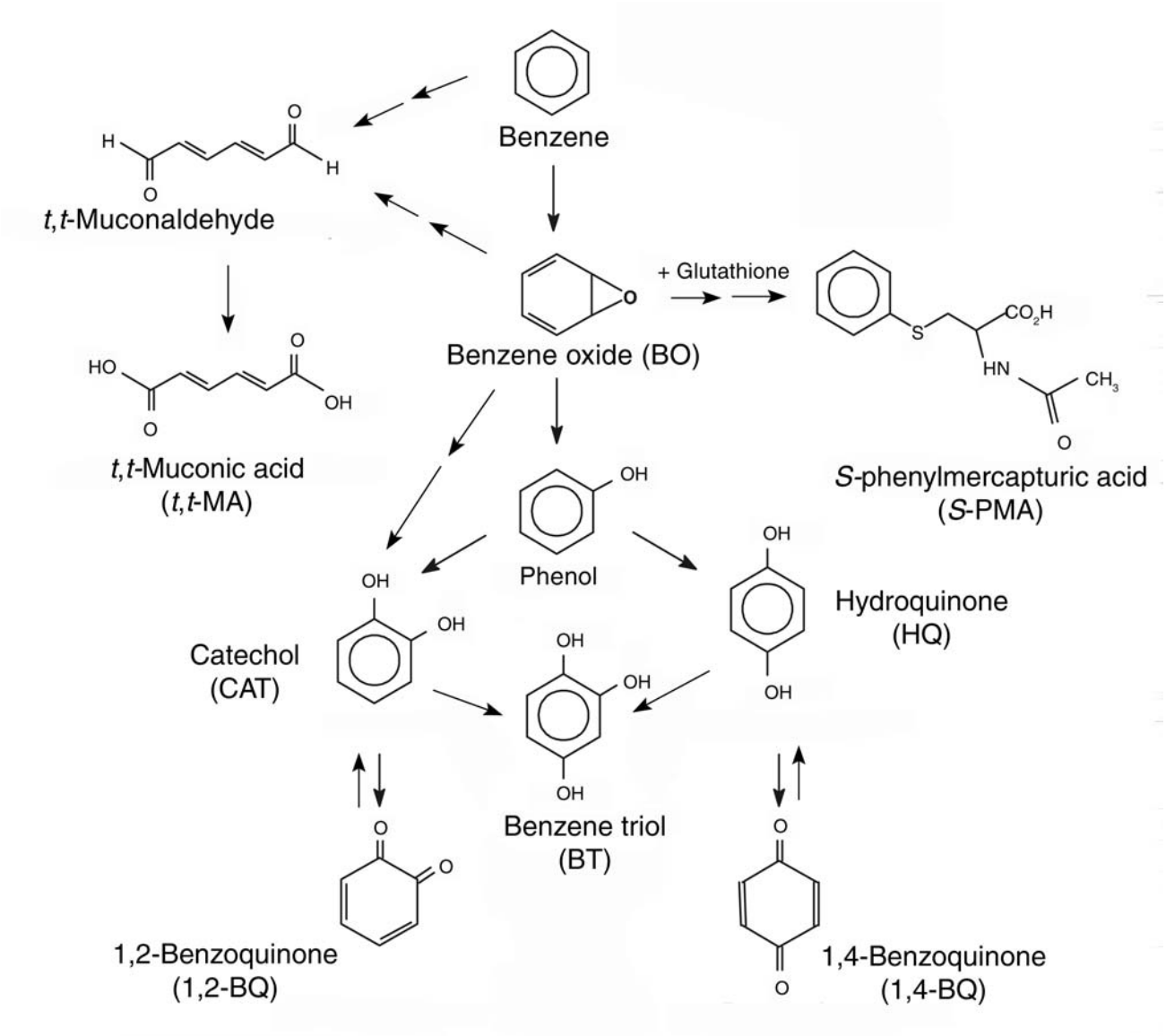
* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

[†] Dr Qu's study, *The Validation of Biomarkers in Humans Exposed to Benzene*, began in September 1996. Total expenditures were $980,580. The draft Investigators' Report from Qu and colleagues was received for review in November 2000. A revised report, received in March 2002, was accepted for publication in May 2002. 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 the Review Committee's Commentary.

pancytopenia (decreased numbers of all blood cell lineages), aplastic anemia, and acute myeloid leukemia (reviewed in Goldstein and Witz 2000).

Benzene needs to be metabolized to induce toxic effects (Snyder and Hedli 1996). Although some benzene metabolism takes place in the target organ, the bone marrow, the primary metabolism site is the liver. The Commentary Figure depicts the major pathways of benzene metabolism and the metabolites formed. The principal mechanism of benzene metabolism in the liver is through oxidation, and the initial steps are catalyzed primarily by the cytochrome P450 2E1 (CYP2E1) isoform—one of the family of cytochrome P450 monooxygenases. One of the key early intermediates is benzene oxide (BO), a highly reactive epoxide that can spontaneously rearrange to form phenol or can be converted via epoxide hydrolase to the dihydrodiol, which is dehydrogenated to form catechol (CAT, 1,2-dihydroxybenzene). Phenol in turn can be oxidized by cytochrome P450 enzymes to CAT, hydroquinone (HQ, 1,4-dihydroxybenzene), or benzene triol (BT, 1,2,4-trihydroxybenzene). BO can react with glutathione to form *S*-phenylmercapturic acid (*S*-PMA) and can undergo opening of the benzene ring, producing metabolites that include *trans,trans*-muconic acid (*t,t*-MA).



Commentary Figure. Pathways of benzene metabolism. Two arrows in a row indicate multiple steps.

In the bone marrow, ring-hydroxylated benzene metabolites are further oxidized to highly reactive quinones, such as the benzoquinones (BQs) 1,2-BQ and 1,4-BQ, which are hematotoxic and genotoxic (Eastmond et al 1987; Smith et al 1989). The highly reactive electrophilic metabolites of benzene, such as BO and the quinones, can form covalent adducts with nucleophilic sites on DNA and circulating proteins such as albumin and hemoglobin.

Several mechanisms of toxicity have been proposed. One widely held hypothesis is that certain benzene metabolites, including BQs, HQ, and products of BT, produce reactive oxygen species that interact with cellular macromolecules (Smith 1996). Eastmond and colleagues have shown that activated benzene metabolites inhibit the activity of the nuclear enzyme topoisomerase II, which is involved in stabilizing the chromosomal structure (Chen and Eastmond 1995). Inhibiting the activity of topoisomerase II may lead to chromosomal abnormalities (Chen and Eastmond 1995; Eastmond et al 2001), and damage or alterations to chromosomes are thought to lead to cancer (Solomon et al 1991; Sorsa et al 1992; Hagmar et al 1998). Some results suggest that a combination of ring-hydroxylated metabolites of benzene compounds may be responsible for benzene's toxic effects (Eastmond et al 1987). An alternative hypothesis is that the precursor of *t,t*-MA, *t,t*-muconaldehyde, possibly in combination with other metabolites, is the toxic product of benzene metabolism (Witz et al 1996) and may induce oncogene expression (Ho and Witz 1997).

BIOMARKERS OF BENZENE EXPOSURE AND EFFECT

Results from studies of workers and of nonhuman species exposed to benzene have suggested many biochemical or pathological responses that might serve as biomarkers of benzene exposure or effect. These responses fall into the broad categories of changes in peripheral blood cell numbers, appearance of metabolites in urine and blood, and chromosomal aberrations. The US National Cancer Institute (NCI) and the Chinese Academy of Preventive Medicine are conducting a long-term, ongoing study of tumor incidence in a cohort of more than 70,000 workers occupationally exposed to benzene in 12 Chinese cities (referred to hereafter as the *NCI/China study*; Hayes et al 1997). They, as well as others, have contributed recent information on biochemical and pathological changes that occur in benzene-exposed workers.

Many occupational studies have reported decreased peripheral red blood cells (RBCs) and white blood cells (WBCs) in workers exposed to benzene, but the decreases varied in extent (eg, Aksoy et al 1971; Kipen et al 1989; Ward et al 1996; reviewed in CONCAWE 1996). In a recent study, workers in Shanghai, China, exposed to high levels of benzene (above 31 ppm), showed decreased numbers of total WBCs, with the major effect on lymphocytes; no changes in polymorphonuclear leukocyte (PMN, also known as *granulocyte*) numbers were noted (Rothman et al 1996). Some studies in which workers had been exposed to benzene at the low end of the occupational range (less than 5 ppm) did not find effects on RBC or WBC numbers (Tsai et al 1983; Collins et al 1991, 1997).

Other studies have indicated that urinary levels of the minor metabolites of benzene, *S*-PMA and *t,t*-MA, may be useful biomarkers of benzene exposure (Bechtold et al 1991; van Sittert et al 1993; Boogaard and van Sittert 1995). *S*-PMA appeared particularly promising because urinary levels in the absence of known exposure to benzene were low and because changes in *S*-PMA level induced by low-dose benzene exposures (down to 0.1 to 1.0 ppm) were detected. Changes in urinary *t,t*-MA were also detectable after low-dose benzene exposure. One drawback of using *t,t*-MA as a benzene biomarker, however, is that *t,t*-MA is not specific for benzene exposure: it also can be formed from sorbitol, which is used as a food preservative (Ruppert et al 1997; Pezzagno et al 1999).

Early studies investigated the suitability of phenol, a major metabolite of benzene, as a biomarker of benzene exposure (eg, Roush and Ott 1977; Inoue et al 1986). Because other sources, such as the metabolism of aromatic amino acids, can produce phenol, however, it is also not a specific marker for low-level exposure to benzene. In addition, phenol levels change only with exposure to benzene concentrations above 10 ppm, so it is not a particularly sensitive marker for exposure. Other urinary metabolites—CAT, HQ, and BT—have not been as extensively studied as phenol, from which they derive. The studies that have been performed, however, indicate that as markers of exposure to benzene, these metabolites have the same drawbacks as phenol (Inoue et al 1988, 1989; Ong et al 1996).

Some studies have also indicated that blood levels of covalent adducts of benzene metabolites coupled to proteins such as albumin or hemoglobin may be suitable biomarkers of exposure to benzene. Bechtold and colleagues measured levels of *S*-phenylcysteine, derived from the reaction of BO and albumin (Bechtold et al 1992a,b). Rappaport and colleagues assayed hemoglobin and albumin adducts of BO (Yeowell-O'Connell et al 1996, 1998). More recently, these investigators have described albumin adducts of BQ as biomarkers of benzene exposure among workers in the NCI/China study and in rats (Waidyanatha et al 1998; Yeowell-O'Connell et al 2001). Measuring levels of blood adducts should provide different information than measuring levels of urinary metabolites because the urinary metabolites of benzene have half-lives measured in hours,

but albumin and hence benzene adducts of albumin in blood have half-lives of approximately 20 days (Allison 1960; Peters 1970).

Standard cytogenetic assays of chromosomal aberrations in workers and in rodents exposed to benzene may also be determinants of subsequent health effects (Forni 1979; Major et al 1994; Tompa et al 1994; Turkel and Egeli 1994; see Eastmond et al 2001). Thus, such chromosomal changes may be thought of as biomarkers of longer-term exposure (integrating damage over months to years) or as early biomarkers of benzene effect. A molecular cytogenetic technique known as *fluorescence in situ hybridization* (FISH) has also been used to detect chromosomal alterations resulting from benzene exposure in humans and other species (Gray and Pinkel 1992; Rupa et al 1995; Zhang et al 1996). Prior to the current study, Dr Martyn Smith and colleagues used this approach in the NCI/China study to detect numerical changes in chromosome 9 in cells from workers in Shanghai exposed to high benzene levels (Zhang et al 1996).

RELATION OF HEI RFA 93-1 BIOMARKER STUDIES TO CURRENT STUDY

Before 1993, assays of possible benzene biomarkers in the urine and blood were frequently time-consuming and lacked the sensitivity to detect low levels of minor metabolites. For example, early assays of blood adducts took several days and could process only a small number of samples. HEI's RFA 93-1 supported studies to develop reliable and sensitive assays for biomarkers of benzene exposure—both recent and longer-term—and of benzene effect. Specifically, from this RFA, HEI funded *Dr Assieh Melikian* and colleagues, American Health Foundation, Valhalla, New York, to develop assays for urinary metabolites; *Dr William Bechtold,* Lovelace Respiratory Institute, Albuquerque, New Mexico, to develop assays for blood adducts; and *Dr David Eastmond*, University of California, Riverside, to develop assays for chromosomal alterations. The results of these studies suggested that one or more of these assays might be useful for detecting benzene exposure or effect (Melikian et al 1999a,b; Eastmond et al 2001). How practical or reliable they would be in a large epidemiologic study of benzene exposure, particularly at exposure levels in the low occupational range of 5 ppm or lower, would be evaluated in the current study.

TECHNICAL EVALUATION

STUDY DESIGN

Study Aims

The Qu study had 2 major aims. The first was to evaluate and validate in a small group of workers (occupationally exposed to high levels of benzene) the usefulness of 3 groups of biomarkers—urinary metabolites, blood protein adducts, and chromosomal aberrations—identified under the aegis of RFA 93-1. Workers were identified in factories in Tianjin, China; they formed part of the cohort of more than 70,000 benzene-exposed workers identified in the NCI/China study (Rothman et al 1997). The second aim was to evaluate relations between exposure to benzene and levels of validated biomarkers in a large cohort of the Tianjin workers. This second group was exposed to a wide range of benzene concentrations, but most had exposures at the lower end of the range (at or below 5 ppm).

The study was divided into 2 phases that reflected the major aims.

Phase 1: Biomarker Validation

Between March 23 and April 28, 1997, Qu and colleagues conducted personal exposure monitoring and collected samples from 25 workers exposed to high levels of benzene (mean current-day exposure, 31.2 ppm) and 25 unexposed workers. The investigators evaluated levels of urinary metabolites, blood adducts, and chromosomal aberrations. Dr Melikian, who developed sensitive assays in her RFA 93-1 study, was the collaborator principally responsible for measuring the urinary benzene metabolites in the current study: *S*-PMA, *t,t*-MA, HQ, CAT, BT, and phenol.

After discussions with HEI, Dr Stephen Rappaport, University of North Carolina, replaced Dr Bechtold in measuring blood adducts (BO and BQ adducts of albumin) in the current study. Rappaport and colleagues had described a simple, rapid, and analytically robust assay for benzene adducts while Bechtold's study was in progress (Yeowell-O'Connell et al 1996).

Dr Eastmond and colleagues were again responsible for assays of chromosomal alterations, which they measured in peripheral blood PMNs and lymphocytes and in vitro activated lymphocytes. They used fluorescent probes specific for chromosome 1 to compare the results with those obtained with these reagents in the prior HEI-funded study (Eastmond et al 2001). They also used specific fluorescent probes to evaluate changes affecting chromosome 7 because investigators from the NCI/China study had

reported aneuploidy (changes in chromosome number) for chromosome 7 at low occupational benzene exposures (Zhang et al 1998.)

Phase 1 comprised 3 parts, described in Figure 4 of the Investigators' Report (all subsequent references to figures and tables are to those in the Investigators' Report). The first, *preliminary sensitivity assessment,* determined whether the assays of prospective biomarkers showed differences between samples from highly exposed workers and those from unexposed control subjects. Urine samples were obtained before and after work, and a blood sample was taken after work.

The second, a substudy to determine *interindividual and intraindividual variability* (also called the *3-Monday study*), evaluated the variability in putative biomarker levels among different individuals and in the same individual on different days. Eleven of the 25 exposed workers who had participated in the preliminary sensitivity assessment gave samples (urine before and after work, blood after work) on 3 consecutive Mondays. Each participant's benzene exposure was measured during the workday on each Monday.

The third part was a *time course study* to determine the half-lives of benzene metabolites in urine. Seven of the 25 benzene-exposed workers in the preliminary sensitivity assessment (and different from the 11 subjects who participated in the interindividual and intraindividual variability substudy) and 4 other subjects provided urine samples before work on a Friday morning, after work on Friday, and at 2 subsequent times (either 16 or 40 hours later and 64 hours later). In this way, urinary benzene metabolite levels were measured at the end of the workweek and over the weekend, when the workers were not exposed occupationally to benzene. Each participant's benzene exposure was measured over the course of the workday on Friday.

Phase 2: Exposure-Response Studies

Between March 12 and April 21, 1998, Qu and colleagues conducted personal exposure monitoring and collected samples from 26 unexposed workers and 105 benzene-exposed workers in the Tianjin area. The investigators focused on subjects exposed to low occupational levels (mean current-day exposure, 4.6 ppm; 30 subjects had benzene exposures of 1 to 5 ppm, and 40 subjects had exposures less than 1 ppm). The investigators evaluated relations between levels of exposure to benzene and levels of biomarkers validated in phase 1 of the study. They also evaluated the numbers of RBCs and of different types of WBCs.

The urinary biomarkers measured in phase 2 were *S*-PMA, *t,t*-MA, and phenol. HQ, CAT, and BT levels, measured in phase 1,were not measured in phase 2 because the results of the phase 1 assays indicated that these metabolites were not sensitive indicators of benzene exposure. Both BO and BQ adducts of albumin in blood were again measured in phase 2. The results of chromosomal aberrations measured by FISH in phase 1 indicated little effect from occupational benzene exposure (see Results). Therefore, Eastmond also used conventional cytogenetic techniques to evaluate aberrations in metaphase chromosomes (found in dividing cells) in samples from both phase 1 and phase 2 subjects.

Study Subjects and Their Occupational Exposures to Benzene

Qu and colleagues recruited workers occupationally exposed to benzene in 4 factories in and around Tianjin: 2 shoe factories, a glue factory, and a sporting goods factory. Prior to the study, the investigators established the major sources of occupational exposure to benzene in each factory. They found that workers were exposed at multiple stages of production and distribution. None of the factories had mechanical general ventilation. In addition, none of the workers used gloves or respirators. The text and Figures 1 through 3 of the Investigators' Report describe the characteristics of each factory and the ways in which workers were exposed to benzene in the factories. As controls, workers not occupationally exposed to benzene were recruited from a nearby food processing plant (phase 1) and a flour factory (phase 2).

The investigators used a 3-step recruitment procedure: (1) A questionnaire was given to all exposed workers, and personnel officers from each factory reviewed the answers. (2) A physical examination and a liver function test were given to workers who had at least 3 years of exposure to benzene, had no known disease, and were not taking medication. (3) An averaged personal benzene exposure was obtained by monitoring an individual at least 3 times during 1 month. Workers with blood cell counts below the normal range were not included in the study.

METHODS

Evaluating Personal Exposure

Qu and colleagues used organic vapor monitors to sample personal exposures to benzene, toluene, and xylene. They measured the latter compounds because workers are frequently exposed to them simultaneously when they are exposed to benzene (for example, in the glue factory, the benzene used to dissolve rubber contained 5% toluene) and these compounds may inhibit benzene metabolism. The investigators monitored exposure by area sampling and by sampling from duplicate personal exposure monitors worn by all participants. To check reliability of

information from the personal monitors, one sampler from a duplicate set was analyzed in China and the other at New York University. The investigators used 3 different measures of each worker's benzene exposure:

Current-day exposure sampling was conducted on the day when biological samples were collected. The investigators used this type of exposure monitoring to correlate same-day benzene exposure with levels of urinary metabolites.

A *4-week mean exposure* was calculated from the mean of personal exposure levels monitored 3 times at 1-week intervals for the month before the investigators collected biological samples and on the day of sample collection. This 4-week mean was used to correlate benzene exposure with levels of albumin adducts.

Lifetime cumulative exposure was estimated by Qu and colleagues using a modification of a previously published method to construct personal retrospective benzene exposures (Dosemeci et al 1994). In brief, the lifetime cumulative benzene exposure for each individual was estimated from individual work history and exposure records available at each factory. These values were used to compare benzene exposure with chromosomal alterations.

Preparing and Handling Biological Samples

The investigators collected urine samples of approximately 50 mL and transferred them at 4°C to the local research institute in China: in phase 1, the Tianjin Institute of Occupational Medicine; and in phase 2, the National Institute of Occupational Medicine. These samples were stored at −20°C and shipped in dry ice to Melikian's laboratory in the United States.

Qu and colleagues collected 10-mL peripheral blood samples, half in heparin-containing tubes (mainly for chromosomal aberrations assays), the other half in citrate-containing tubes (for total cell number and protein adduct assays). The investigators maintained samples at 4°C until they were processed at the local research institute, within 3 hours of collection. Plasma was separated from cells by centrifugation, frozen at −20°C, and shipped in dry ice to Rappaport's laboratory.

For cytogenetic analysis of peripheral blood WBCs, the investigators removed RBCs and fixed the remaining cells, predominantly PMNs and resting lymphocytes, in methanol plus acetic acid. Fixed cells were stored at 4°C and transported at room temperature to Eastmond's laboratory, where they were dropped onto glass slides. Eastmond and colleagues similarly prepared slides of fixed activated lymphocytes after culturing whole blood with phytohemagglutinin for 51 hours (optimal for evaluating chromosome breakage) or 72 hours (optimal for aneuploidy). To capture dividing cells in metaphase, they added colcemid to cultures of activated lymphocytes 3 hours prior to harvesting at 51 hours. Fixed, activated lymphocytes were stored at −20°C until being transported to the United States at room temperature.

Evaluating Blood Cell Numbers and Cell Types

Using an electronic cell counter in the local research institute, Qu and colleagues determined total numbers of WBCs, RBCs, and platelets in each blood sample. In addition, 5 aliquots of each sample were smeared onto glass slides, air dried, and stored at −20°C until they were shipped to the United States at room temperature. Quest Diagnostics, a commercial clinical laboratory in the United States, evaluated the numbers and types of different WBCs—the differential cell count—on the slides. They counted a total of 900 WBCs for each individual (separate blinded evaluations of 100, 400, and 400 cells on different slides). The predominant WBC populations present were lymphocytes, monocytes, and PMNs, which comprised mainly neutrophils with smaller numbers of eosinophils and basophils.

Separating and Analyzing Urinary Benzene Metabolites

Melikian and colleagues used liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ES-MS/MS) to simultaneously measure the urinary concentrations of *S*-PMA and *t,t*-MA, to separately measure the concentrations of HQ and CAT, and to estimate the concentration of BT (Melikian et al 1999a,b). Phenol was measured by gas chromatography coupled to mass spectrometry. They also measured urinary levels of creatinine as a reference standard for the concentration of metabolites in samples and cotinine, one of the major metabolites of nicotine, as an indicator of smoking status (Melikian et al 1994). Because some study subjects who described themselves as nonsmokers had high cotinine levels, the investigators designated only individuals with cotinine levels less than 100 µg/g creatinine as nonsmokers.

Separating and Analyzing Blood Albumin Adducts

Rappaport and colleagues isolated albumin from plasma using the method described by Lindstrom and colleagues (1998). Cysteinyl adducts of BQ and BO with albumin were measured by gas chromatography coupled to mass spectrometry after reacting vacuum-dried samples with trifluoroaceticanhydride and methanesulfonic acid (Waidyanatha et al 1998). Using this technique, the investigators reported that they could reliably measure BO adducts and the 1,4-BQ adducts, but not the apparently less stable 1,2-BQ adducts of albumin.

Assessing Chromosomal Alterations

Eastmond and colleagues evaluated chromosomal alterations in the nuclei of peripheral blood cells in interphase (PMNs and unstimulated lymphocytes) and in metaphase (in vitro activated lymphocytes) using the following techniques.

Eastmond and associates used their previously described FISH approach with tandem-labeled probes to evaluate benzene-induced alterations in chromosome 1 (Hasegawa et al 1995; Rupa et al 1995). They used probes specific for the adjacent α- and classical-satellite regions—sections of repetitive DNA sequences close to the centromere that are found on all chromosomes. To evaluate changes in chromosome 7, Eastmond and colleagues evaluated 1000 cells per individual with a fluorescent probe specific for the centromeric α-satellite sequences of this chromosome. They included positive controls for chromosomal breakage (2 Gy radiation) and hyperdiploidy (diethylstilbestrol treatment) in evaluations of peripheral blood lymphocytes and PMNs.

To evaluate chromosomal aberrations in colcemid-treated, activated lymphocytes, Eastmond and colleagues scored 100 cells in metaphase per individual. To assess structural alterations, they scored metaphase cells containing 45 or more chromosomes; to assess chromosome number, they evaluated the number of metaphase spreads containing more than or fewer than 46 chromosomes (specifically, ≤ 44, 45, or > 47 chromosomes, and polyploidy).

Statistical Analyses

In phase 1, Qu and colleagues used a trend test to assess the sensitivity of a variety of biomarkers to benzene exposure by comparing measurements made in 3 groups of workers: the unexposed, those exposed to less than 30 ppm benzene, and those exposed to at least 30 ppm benzene. In addition, to assess the variability of putative biomarkers within subjects evaluated at different times and between subjects evaluated at the same time, the investigators used an analysis that adjusted for the variability in benzene exposures, which varied widely for different individuals and for the same individual at different times.

Qu and colleagues used several statistical methods to investigate the exposure–biomarker response relations that were the major focus of the report. For these analyses, phase 1 and phase 2 data were combined and grouped by exposure levels. Some data were compared by 1-way analysis of variance (for example, Figures 14–16), some by unadjusted regression (*S*-PMA on benzene in Figures 17–19), and multiple regression of biomarkers, adjusting for potential confounders such as age, smoking, and gender (summarized in Tables 15 and 16). After careful consideration (described in Appendix A), the investigators included unexposed subjects in the multiple regressions, but not in some of the unadjusted regressions (for example, in the data shown in Figures 17 and 18).

RESULTS

Range of Worker Exposure to Benzene

On the day of sample collection, benzene exposures ranged from 0.06 to 122 ppm (median, 3.2 ppm). The 25 phase 1 participants had high mean current-day benzene exposure (31.2 ppm), with lower exposures to toluene (mean, 2.9 ppm) and xylene (mean, 0.11 ppm) (Table 4). In the substudy evaluating interindividual and intraindividual variability, benzene exposure for each of the 11 participants varied considerably from week to week in a range of 4.8 to 118.3 ppm. Mean current-day exposures for the 11 participants on each of the 3 Mondays when benzene exposure was monitored were 37.0, 16.8, and 20.5 ppm.

In phase 2 (Table 6), the mean current-day benzene exposure of 105 subjects—selected to have a broader range of exposures—was 4.6 ppm; their 4-week mean exposure was 5.2 ppm; and their lifetime cumulative mean exposure was 53.8 ppm-years. Toluene levels were much higher than in phase 1 (current-day mean, 33.1 ppm; 4-week mean, 26.3 ppm) and were higher than the average benzene exposures in phase 2. Xylene levels were low (current-day mean, 0.40 ppm).

Decreased Peripheral Red and White Blood Cell Counts

The numbers of RBCs, total WBCs, and neutrophils in blood showed an exposure-related decrease, using benzene exposure levels averaged over 4 weeks, in exposed workers compared with unexposed controls (Figure 8). Blood cell counts in the exposed workers were still in the normal range, however. This analysis did not find an association between benzene exposure and numbers of lymphocytes or other WBCs. Regression analyses showed significant negative associations between benzene exposure and cell counts of RBCs, WBCs, and neutrophils. After adjusting for confounders, including age, sex, cotinine level (ie, smoking status), and toluene exposure, weak negative associations were found between 4-week benzene exposure and decreased lymphocyte and monocyte counts. The investigators found indications that "smoking may have masked the association between benzene and lymphocyte count in unadjusted analyses" (analyses not shown).

Qu and colleagues also reported decreases in WBC and neutrophil numbers with increasing estimated lifetime cumulative exposure to benzene (Figure 9). RBC numbers were decreased by a similar amount in all groups with different lifetime cumulative exposures to benzene, indicating

that the effect on RBCs was not dependent on cumulative exposure (Figure 9). Regression analyses that adjusted for confounders indicated strong negative associations with RBCs, WBCs, neutrophils, and monocytes, and weaker associations with lymphocytes and eosinophils. Decreased neutrophil and RBC numbers were associated with *duration* of exposure to benzene (Figure 10), whereas decreased RBC, WBC, neutrophil, and eosinophil numbers were associated with *intensity* of exposure to benzene, expressed as average exposure concentration per year (Figure 11).

S-PMA: Most Sensitive and Specific of Urinary Biomarkers Tested

The investigators found very low levels of *S*-PMA in the urine of unexposed subjects, with many values at the limit of detection. *S*-PMA levels were much higher in exposed workers, particularly after work. This workday increase in *S*-PMA levels correlated with current-day measured benzene exposure (Table 10). In addition, *S*-PMA levels correlated with benzene exposure over a wide range, even in workers exposed to benzene levels at or below 0.5 ppm (Figures 12 and 14, and Table 16). The multiple regression analyses in Tables 15 and 16 show that the differences between measures of *S*-PMA levels before and after work generally had weaker associations with exposure than the separate measures themselves.

Changes in *t,t*-MA levels showed a pattern similar to changes in *S*-PMA levels: namely, levels were low in the urine of unexposed subjects and increased with increasing benzene exposure (Figures 13 and 15). After-work levels of *t,t*-MA in workers exposed to 1 ppm were higher than levels in control subjects, but those in workers exposed to no more than 0.5 ppm benzene were not (Table 16). Background levels of *t,t*-MA were higher than those of *S*-PMA (Table 14).

In benzene-exposed workers, changes in levels of HQ, CAT, and phenol in urine were dependent on benzene exposure over the workday. However, background levels of these metabolites were high, and the concentrations of these metabolites in urine increased only with benzene exposure above 5 ppm. No correlation was found between urinary BT concentration and benzene exposure.

Multiple regression analyses of urinary metabolites on benzene exposure indicated that toluene exposure, smoking status, and cotinine levels had no significant effects on urinary metabolites. The investigators calculated that the half-lives of all benzene metabolites in urine were relatively short, approximately 14 hours.

Albumin Adduct Markers of Recent Response

Because the half-life of albumin in blood is approximately 20 days, Qu and colleagues correlated albumin adduct levels with 4-week mean benzene exposures rather than the current-day exposures they used with urinary metabolites. Levels of the adduct of BO and albumin (BO–Alb) and the adduct of 1,4-BQ and albumin (1,4-BQ–Alb) correlated with 4-week mean benzene exposure in exposed workers (Figure 23), but were also relatively high in unexposed subjects. Adduct levels were not influenced by smoking, but BO–Alb levels did vary by age (Table 21), and 1,4-BQ–Alb levels varied by age and sex (decreased with age, increased in men, Table 23).

Chromosomal Aberration Markers of Long-Term Response

Using conventional cytogenetic analysis of all chromosomes, the investigators found chromosomal aberrations more often in benzene-exposed workers compared with unexposed workers (Table 27); they noted a trend in the exposure-response pattern (based on 4-week mean benzene exposure levels, Table 27). The pattern was unusual, however, because frequencies of chromosomal aberrations in the lowest exposure group (at or below 0.5 ppm) were higher than those in unexposed controls but similar to those in the highest exposure group (at or above 30 ppm) (Tables 27 and 29). Exposed workers had increased chromosomal loss and a 2-fold increase in breakage of chromosomes compared with controls. No differences in hyperdiploidy in peripheral blood cells of exposed and control populations were detected.

Using the fluorescence in situ hybridization (FISH) analysis of chromosomes 1 and 7 in phase 1, the investigators found few significant differences between chromosomal aberrations in cells from benzene-exposed workers compared with cells from control subjects. In addition they found no increases in numerical or structural changes in lymphocytes activated in vitro, a small but not statistically significant increase in hyperdiploid cells in unstimulated lymphocytes, and in activated lymphocytes a *lower* frequency of breakage in the region of the chromosome that bound the tandem probes, 1cen-1q12. Using the same technique in a follow-up study on a subset of the phase 2 subjects, however, the investigators reported finding a significant exposure-related increase in breakage in the 1cen-1q12 region in activated lymphocytes from benzene-exposed workers as compared with controls (Appendix Table B.11 of the Investigators' Report).

DISCUSSION

This study has made important contributions to the literature regarding the utility of possible biomarkers of benzene exposure in occupational settings. It is the first to evaluate multiple possible biomarkers across a wide range of exposures, and to show effects at the lowest end of this range. In addition to using sensitive assays for the biomarkers, Qu and colleagues made great efforts to accurately measure and monitor personal exposure to a wide range of benzene levels in the workplace—critical features for assessing the accuracy of biomarker information.

A key positive feature of the study design was Qu's use of a 2-step approach: phase 1, to determine whether previously described putative benzene biomarkers could be used in a small-scale epidemiologic study; and phase 2, to examine the relation between level of benzene exposure and concentrations of the validated biomarkers in a larger study using the information obtained in phase 1. In addition, Qu and colleagues paid careful attention to quality control issues. For example, they implemented procedures to ensure safe handling of samples before they were analyzed; prepared blanks, spiked samples, and aliquots of pooled samples to evaluate the method; and rigorously compared measurements made in Chinese and US laboratories.

One potentially important finding was the association between decreases in the numbers of circulating red and white blood cells (albeit remaining within the normal range) and increases in benzene exposure. Decreases in blood counts were even reported at the lowest end of the exposure range, 0.5 ppm or lower. The decreases in RBC numbers confirm the results of several other studies (Kipen et al 1989; Rothman et al 1996; Khuder et al 1999), although some other studies did not find associations between RBC numbers and low-level benzene exposure (Tsai et al 1983; Collins et al 1991, 1997).

Qu and colleagues found WBC decreases associated with benzene exposure, as have others (eg, Kipen et al 1989; Rothman et al 1996). However, Qu and colleagues' findings differ in that the predominant WBC affected by benzene was the neutrophil, with much weaker effects on lymphocytes. By contrast, the study by Rothman and colleagues of benzene-exposed workers in China did not find an effect on neutrophil number but did find decreased lymphocyte numbers (Rothman et al 1996). In that study, as well as in earlier occupational studies reporting changes in lymphocyte numbers as sensitive indicators of benzene exposure (eg, Kipen et al 1989), the average exposures to benzene were much higher than those in the current study. Qu and colleagues' findings suggest that a decrease in the number of neutrophils may be a very sensitive marker of occupational benzene exposure. This is of particular interest because benzene exposure is associated with the development of leukemias with myeloid lineage precursors in the bone marrow. However, a plausible biological link between benzene targeting bone marrow and sustained decreases in circulating levels of neutrophils—short-lived, terminally differentiated cells—is not clear. One explanation may be that exposure to benzene slows differentiation of neutrophil precursors into mature cells by a small amount. Further work is required to corroborate the decrease in neutrophil numbers. If the results are confirmed, research would be needed to show how this change may be linked mechanistically to benzene effects on precursor cells.

Qu and colleagues' findings of low background levels of *S*-PMA in unexposed individuals and large changes in *S*-PMA levels over the work shift in exposed individuals corroborate and extend previous studies indicating that urinary *S*-PMA is a sensitive and specific biomarker of recent benzene exposure (Boogaard and van Sittert 1995; Melikian et al 1999a,b). For *t,t*-MA, the current study's findings of low background levels in unexposed workers and exposure-related changes in levels over the work shift in benzene-exposed individuals suggest that this metabolite may also be a useful marker of recent benzene exposure. In common with other studies, however, Qu and colleagues found higher levels of *t,t*-MA than *S*-PMA in unexposed individuals (Boogaard and van Sittert 1995; Melikian et al 1999a,b), most likely resulting from the metabolism of sorbitol in food to *t,t*-MA (Ruppert et al 1997; Pezzagno et al 1999). Thus, as Qu and colleagues point out, the specificity of *S*-PMA to benzene exposure, the low *S*-PMA background levels in unexposed individuals, and the detection of changes in *S*-PMA levels even when benzene exposure was low are important reasons for considering *S*-PMA to be the most useful urinary marker of benzene exposure.

The results of the current study also confirm previous findings indicating that some urinary metabolites of benzene have little use as biomarkers of benzene exposure (Boogaard and van Sittert 1995; Melikian et al 1999a,b). Changes in HQ, CAT, and phenol levels were associated with benzene exposure level, but only at levels above 5 ppm. Taken in conjunction with the high background levels in control subjects, confirming that these molecules are derived from pathways other than occupational exposure to benzene, it may be concluded that HQ, CAT, and phenol are not reliable markers of low-level occupational benzene exposure. The investigators also found that BT was not a useful marker of benzene exposure because levels of BT were not associated with levels of benzene exposure.

Qu and colleagues' findings of benzene-related changes in the concentrations of BO–Alb and 1,4-BQ–Alb suggest

these albumin adducts may be reasonable markers of exposure to benzene. As was found in an earlier study (Yeowell-O'Connell et al 1998), however, the investigators found relatively high background levels in unexposed subjects. Thus, the suitability of these adducts as biomarkers is uncertain. To be able to use the adducts as benzene biomarkers, further studies are necessary to clarify how they are generated in individuals not exposed to benzene. Because phenol and HQ—the precursors of 1,4-BQ—can be formed from sources other than benzene exposure, they are likely to contribute to the benzene-independent formation of the 1,4-BQ–Alb adduct. Endogenous sources of BO–Alb are not known, however. The investigators speculate that this adduct may result from free-radical–mediated reactions of simple aromatic molecules.

The investigators recently reported an additional analysis of albumin adduct levels in the same set of workers but including 4 additional benzene-exposed individuals who were not part of the HEI study (Rappaport et al 2002). Using a different analytical approach, they found that the relation between adduct levels and benzene exposure was less than linear with benzene levels above 1 ppm. The investigators interpreted this finding to indicate that benzene metabolism by CYP2E1 was saturable, starting around 1 ppm benzene. The investigators also found that absolute levels of the adducts increased with smoking and decreased with age. Further, the relative proportions of 1,4-BQ–Alb and BO–Alb adducts were affected by age, toluene exposure, and alcohol, suggesting that many factors influence CYP2E1 metabolism of benzene (Rappaport et al 2002).

Qu and colleagues' findings of benzene-induced chromosomal aberrations using conventional cytogenetic approaches confirm results from many previous studies (reviewed in Zhang et al 2002). The findings obtained using the tandem-labeling FISH technique to evaluate aberrations on specific chromosomes in phase 1 of the current study were generally negative. Thus, this approach may also be limited as a biomarker assay for occupational exposure to benzene. However, in the follow-up study on a subset of phase 2 subjects exposed to low levels of benzene, Qu and colleagues found an exposure-related increase in breakage in the 1cen-1q12 region in interphase nuclei, suggesting that the tandem-labeling FISH approach may be capable of detecting chromosomal breaks. Similar findings were reported in a small study of workers exposed to low levels of benzene in Estonia (Marcon et al 1997; Eastmond et al 2001). Consistent with previous studies (Eastmond et al 2001), data from the current study indicate that FISH does not detect aberrations at higher exposures. Why the tandem-labeling approach appears to detect chromosomal aberrations at low levels of exposure, but not at high levels, is not clear but augurs badly for using the technique in studies of a wide range of exposures.

Previous findings by Smith and colleagues, who used a different FISH approach to evaluate chromosomal aberrations in workers in Shanghai as part of the larger NCI/China study, were somewhat different (Zhang et al 1998, 1999). Using individual, rather than tandem, probes, these investigators found increased hyperploidy and deletions for chromosomes 5 and 7 in one study (Zhang et al 1998) and increased hyperploidy for a number of chromosomes in other analyses (Zhang et al 1999, 2002). Thus, the reported responses in the NCI/China study were more extensive than those described in the current study. Comparison of the results of these studies is difficult, however, as different methods, chromosomal probes, and scoring systems were used. Moreover, although the median exposures of workers in the NCI/China and HEI studies were similar, the workers in the NCI/China study with above-median exposures were exposed to much higher benzene levels than those participating in the HEI study (Rothman et al 1996). These higher exposures in the NCI/China study may also have contributed to the different FISH results obtained in the 2 studies.

In addition, the frequencies of hyperdiploidy detected in the NCI/China study were extremely high, even in control, unexposed subjects. For example, Smith and colleagues reported a frequency of trisomy of approximately 0.5% for one chromosome in control metaphase cells (Smith et al 1998; Zhang et al 1998.) Assuming this frequency held for all chromosomes, it would correspond to an overall frequency of hyperdiploidy of approximately 11.5% (0.5% $\times$ 23 chromosomes). This is well above the mean hyperdiploidy frequency of 0.73% for all chromosomes combined that has been reported for unexposed individuals in several studies (reviewed in Eastmond et al 1995).

Why such high frequencies of hyperdiploidy were found in unexposed as well as benzene-exposed workers in the NCI/China study is not clear. As indicated above, high frequencies of hyperdiploidy were not found in the current study using FISH or conventional cytogenetic approaches. In the few earlier cytogenetic studies that evaluated hyperdiploidy in nonpoisoned benzene workers (Forni et al 1971; Ding et al 1983;Yardley-Jones et al 1990), the combined frequencies of hyperdiploidy for all chromosomes were very low (ranging from 0.1% to 0.4% in the exposed individuals compared with 0% in the control, unexposed populations).

The current study produced some other unexpected findings. First, the investigators found little or no effect of smoking on levels of the urinary benzene metabolites *S*-PMA and *t,t*-MA, whereas other studies have reported 3-fold to

5-fold higher levels in smokers (Boogaard and van Sittert 1995; Ghittori et al 1999; Melikian et al 1999a,b). Interestingly, the investigators found that urinary *t,t*-MA levels did not differ between smokers and nonsmokers in either phase 1 or phase 2 subjects, but *S*-PMA levels were higher in the urine of smokers in phase 1 subjects. The differences in *S*-PMA levels between smokers and nonsmokers were not found, however, when phase 1 and phase 2 results were combined. This finding suggests that the combined analysis of phase 1 and phase 2 may have introduced unmeasured confounding. This is possible in a study in which exposures were analyzed in different years, at different sites, and in different subjects (for example, according to Table 2, phase 2 study subjects were older), with much lower exposure levels—by design—in phase 2 than phase 1. Although Qu and colleagues amply address many aspects of this issue in the report, uncertainty remains about the validity of combining data from phases 1 and 2 of the study.

Qu and colleagues found that the half-life of metabolites in urine was approximately 14 hours, but previous studies have indicated considerably shorter values of approximately 9 hours for *S*-PMA and 5 hours for *t,t*-MA and phenol (Boogaard and van Sittert 1995, 1996). The fact that in the current study specimens were taken infrequently (ie, 16 or 40 hours after the end of the workday) may make the values reported less accurate than those reported previously.

Qu and colleagues found that endpoint outcomes generally correlated well with air levels of benzene in the workplace. However, their job descriptions indicated that many of the factory workers might have had dermal exposure to benzene from benzene-containing products. Qu and colleagues appropriately interpreted the good correlation of endpoints with air levels in the factories to indicate that absorption of benzene via dermal exposure was probably not a major route of exposure in the study.

The investigators also found that mean exposure to toluene was higher than exposure to benzene (xylene exposure levels were very low, at or below 0.5 ppm in phases 1 and 2 of the study). Regression analyses indicated that toluene did not affect the endpoints evaluated, suggesting that exposure to toluene does not affect responses to benzene. Toluene inhibits the metabolism of benzene, however, so this lack of interaction is somewhat surprising (Sato and Nakajima 1979). As indicated above, a more recent analysis, using a different analytical approach and in a slightly different population of workers, showed that albumin adduct levels were affected by toluene (Rappaport et al 2002).

CONCLUSIONS

This study of workers occupationally exposed to benzene has made important contributions to the literature regarding the utility of possible biomarkers of benzene exposure and effect. This is the first study to evaluate multiple possible biomarkers—blood cell counts, urinary metabolites, blood adducts, and chromosomal aberrations—across a wide range of exposures, and to report effects at the lowest end of the exposure range. A further strong point of the study was its 2-step approach: possible biomarkers were validated in a small number of more highly exposed workers before they were applied to a larger population exposed to a wider range of benzene levels, with emphasis on exposures at the low end of the occupational range. In addition, Qu and colleagues made great efforts to accurately measure and monitor personal exposure to a wide range of benzene levels in the workplace—critical features for assessing the validity of biomarker information. The investigators also paid careful attention to quality control issues.

The study's most novel finding was that exposure to benzene was associated with decreased circulating neutrophil numbers, with a smaller effect on lymphocyte numbers. This result suggests the possibility that changes in neutrophil numbers may be a sensitive marker of benzene exposure, but the finding needs to be corroborated because other studies have found changes in lymphocyte, but not neutrophil, numbers.

The study has confirmed and extended previous findings about the usefulness of biochemical and biological responses as biomarkers of exposure to benzene. Of the metabolites examined, urinary *S*-PMA was the best biomarker of short-term exposure to benzene because of its low background level in unexposed individuals, detection at low levels of occupational exposure, half-life of approximately 14 hours, and specificity for benzene exposure. The urinary metabolite *t,t*-MA may also prove useful, but changes in its level were not detectable at the lowest levels of benzene exposure. Further, other studies have shown that it can be produced from sorbitol, which is part of people's food intake. The study also found that the other urinary metabolites measured—phenol, HQ, CAT, and BT—were not useful markers of benzene exposure: exposure-independent levels were high and exposure-dependent changes were small.

Qu and colleagues confirmed that BO–Alb and 1,4-BQ–Alb adducts in blood also may be useful as markers of recent exposure (days to weeks) to benzene. Changes in adduct levels were associated with benzene exposure

level, but adduct levels in the absence of exposure to benzene were quite high. The study also confirmed that increases in chromosomal aberrations, which integrate damage over months to years, are detectable by conventional cytogenetic techniques in occupationally exposed workers compared with controls. Their results also indicate that evaluating aberrations on specific chromosomes using a FISH approach may have limited use as a biomarker of benzene exposure or effect.

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REFERENCES

Aksoy M, Dincol K, Akgun T, Erdem S, Dincol G. 1971. Hematological effects of chronic benzene poisoning in 217 workers. Br J Ind Med 28:296–302.

Albertini RJ, Šrám RJ, Vacek PM, Lynch J, Nicklas JA, van Sittert NJ, Boogaard PJ, Henderson RF, Swenberg JA, Tates AD, Ward JB Jr, Wright M, et al. 2003. Biomarkers in Czech Workers Exposed to 1,3-Butadiene: A Transitional Epidemiologic Study. HEI Research Report 116. Health Effects Institute, Boston MA.

Allison AC. 1960. Turnover of erythrocytes and plasma proteins in mammals. Nature 188:37–40.

Bechtold WE, Lucier G, Birnbaum LS, Yin SN, Li GL, Henderson RF. 1991. Muconic acid determinations in urine as a biological exposure index for workers occupationally exposed to benzene. Am Ind Hyg Assoc J 52:473–478.

Bechtold WE, Sun JD, Birnbaum LS, Yin S-N, Li G-L, Kasicki S, Lucier G, Henderson RF. 1992a. *S*-phenylcysteine formation in hemoglobin as a biological exposure index to benzene. Arch Toxicol 66:303–309.

Bechtold WE, Willis JK, Sun JD, Griffith WC, Reddy TV. 1992b. Biological markers of exposure to benzene: *S*-phenylcysteine in albumin. Carcinogenesis 13:1217–1220.

Boogaard PJ, van Sittert NJ. 1995. Biological monitoring of exposure to benzene: A comparison between *S*-phenylmercapturic acid, *trans,trans*-muconic acid, and phenol. Occup Environ Med 52:611–620.

Boogaard PJ, van Sittert NJ. 1996. Suitability of *S*-phenyl mercapturic acid and *trans,trans*-muconic acid as biomarkers for exposure to low concentrations of benzene. Environ Health Perspect 104(Suppl 6): 1151–1157.

Chen H, Eastmond DA. 1995. Topoisomerase inhibition by phenolic metabolites: A potential mechanism for benzene's clastogenic effects. Carcinogenesis 16:2301–2307.

Collins JJ, Conner P, Friedlander BR, Easterday PA, Nair RS, Braun J. 1991. A study of the hematologic effects of chronic low-level exposure to benzene. J Occup Med 33:619–626.

Collins JJ, Ireland BK, Easterday PA, Nair RS, Braun J. 1997. Evaluation of lymphopenia among workers with low-level benzene exposure and the utility of routine data collection. J Occup Environ Med 39:232–237.

CONCAWE. 1996. Scientific Basis for an Air Quality Standard on Benzene. Conservation of Clean Air and Water in Europe, Brussels, Belgium.

Dosemeci M, Li G-L, Hayes RB, Yin S-N, Linet M, Chow WH, Wang Y-Z, Jiang Z-L, Dai T-R, Zhang W-U, Chao X-J, Ye P-Z et al. 1994. Cohort study among workers exposed to benzene in china: II. Exposure assessment. Am J Ind Med 26:401–411.

Ding X, Li Y, Ding Y, Yang H. 1983. Chromosome changes in patients with chronic benzene poisoning. Chinese Medical J 96:681–685.

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.

Eastmond DA, Smith MT, Irons RD. 1987. An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. Toxicol Appl Pharmacol 91:85–95.

Eastmond DA, Schuler M, Rupa DS. 1995. Advantages and limitations of using fluorescence in situ hybridization for the detection of aneuploidy in interphase human cells. Mutat Res 348:153–162.

Environmental Protection Agency (US). 1998. Carcinogenic Effects of Benzene: An Update. EPA/600/P-97/001F. Office of Research and Development, Washington DC.

Forni A. 1979. Chromosome changes and benzene exposure: A review. Rev Environ Health 3:5–7.

Forni A, Cappellini A, Pacifico E, Vigliani EC. 1971. Chromosome changes and their evolution in subjects with past exposure to benzene. Arch Environ Health 23:385–391.

Ghittori S, Imbriani M, Maestri L, Capodaglio E, Cavalleri A. 1999. Determination of *S*-phenylmercapturic acid in urine as an indicator of exposure to benzene. Toxicol Lett 108:329–334.

Goldstein BD, Witz G. 2000. Benzene. In: Environmental Toxicants: Human Exposures and Their Health Effects, 2nd Ed (Lippmann M, ed), pp 121–149. John Wiley & Sons, New York NY.

Gray JW, Pinkel D. 1992. Molecular cytogenetics in human cancer diagnosis. Cancer 69(Suppl 6):1536–1542.

Hagmar L, Bonassi S, Stromberg U, Brogger A, Knudsen LE, Norppa H, Reuterwall C. 1998. Chromosomal aberrations in lymphocytes predict human cancer: A report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). Cancer Res 58: 4117–4121.

Hasegawa LS, Rupa DS, Eastmond DA. 1995. A method for the rapid generation of alpha- and classical satellite probes for human chromosome 9 by polymerase chain reaction using genomic DNA and their application to detect chromosomal alterations in interphase cells. Mutagenesis 10:471–476.

Hayes RB, Yin SN, Dosemeci M, Li GL, Wacholder S, Travis LB, Li CY, Rothman N, Hoover RN, Linet MS. 1997. Benzene and the dose-related incidence of hematologic neoplasms in China. Chinese Academy of Preventive Medicine–National Cancer Institute Benzene Study Group. JNCI 89:1065–1071.

Ho TY, Witz G. 1997. Increased gene expression in human promyeloid leukemia cells exposed to *trans,trans*-muconaldehyde, a hematotoxic benzene metabolite. Carcinogenesis 18:739–744.

Inoue O, Seiji K, Kasahara M, Nakatsuka H, Watanabe T, Yin SG, Li GL, Cai SX, Jin C, Ikeda M. 1988. Determination of catechol and quinol in the urine of workers exposed to benzene. Br J Ind Med 45:487–492.

Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin S-N, Li G-L, Cai S-X, Jin C, Ikeda M. 1989. Excretion of 1,2,4-benzenetriol in the urine of workers exposed to benzene. Br J Ind Med 46:559–565.

Inoue O, Seiji K, Kasahara M, Nakatsuka H, Watanabe T, Yin SG, Li GL, Jin C, Cai SX, Wang XZ, et al. 1986. Quantitative relation of urinary phenol levels to breathzone benzene concentrations: A factory survey. Br J Ind Med 43:692–697.

Khuder SA, Youngdale MC, Bisesi MS, Schaub EA. 1999. Assessment of complete blood count variations among workers exposed to low levels of benzene. J Occup Environ Med 41:821–826.

Kipen HM, Cody RP, Goldstein BD. 1989. Use of longitudinal analysis of peripheral blood counts to validate historical reconstructions of benzene exposure. Environ Health Perspect 82:199–206.

Lindstrom AB, Yeowell-O'Connell K, Waidyanatha S, McDonald TA, Golding BT, Rappaport SM. 1998. Formation of hemoglobin and albumin adducts of benzene oxide in mouse, rat, and human blood. Chem Res Toxicol 11:302–310.

Major J, Jakab M, Kiss G, Tompa A. 1994. Chromosome aberration, sister-chromatid exchange, proliferative rate index, and serum thiocyanate concentration in smokers exposed to low-dose benzene. Environ Mol Mutagen 23:137–142.

Marcon F, Zijno A, Crebelli R, Carere A, Schuler M, Parks R, Eastmond DA. 1997. Detection of cytogenetic damages in blood cells of benzene-exposed workers by multicolor fluorescence in situ hybridization (FISH). Mutat Res 379:S141.

Melikian AA, Meng M, O'Connor R, Hu P, Thompson SM. 1999a. Development of Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans. Research Report 87. Health Effects Institute, Cambridge MA.

Melikian AA, O'Connor R, Prahalad AK, Hu P, Li H, Kagan M, Thompson S. 1999b. Determination of the urinary benzene metabolites *S*-phenylmercapturic acid and *trans,trans*-muconic acid by liquid chromatography-tandem mass spectrometry. Carcinogenesis 20:719–726.

Melikian AA, Prahalad AK, Secker-Walker RH. 1994. Comparison of levels of urinary benzene metabolite *trans,trans*-muconic acid in smokers and nonsmokers, and the effects of pregnancy. Cancer Epidemiol Biomarkers Prev 3:239–244.

Occupational Safety and Health Administration (US). 1987. Final rule on occupational exposure to benzene. Fed Regist 54:34660–34762.

Ong CN, Kok PW, Ong HY, Shi CY, Lee BL, Phoon WH, Tan KT. 1996. Biomarkers of exposure to low concentrations of benzene: A field assessment. Occup Environ Med 53:328–333.

Peters T. 1970. Serum albumin. Adv Clin Chem 13:37–111.

Pezzagno G, Maestri L, Fiorentini ML. 1999. *trans,trans*-Muconic acid, a biological indicator to low levels of environmental benzene: Some aspects of its specificity. Am J Ind Med 35:511–518.

Rappaport SM, Waidyanatha S, Qu Q, Shore R, Jin X, Cohen B, Chen LC, Melikian AA, Li G, Yin S, Yan H, Xu B, Mu R, Li Y, Zhang X, Li K. 2002. Albumin adducts of benzene oxide and 1,4-benzoquinone as measures of human benzene metabolism. Cancer Res 62:1330–1337.

Rothman N, Li G, Dosemeci M, Bechtold WE, Marti GE, Wang Y, Linet M, Xi L, Lu W, Smith MT, Titenko-Holand N, Zhang L, Blot W, Yin S, Hayes R. 1996. Hematotoxicity among Chinese workers heavily exposed to benzene. Am J Ind Med 29:236–246.

Rothman N, Smith MT, Hayes RB, Traver RD, Hoener B, Campleman S, Li GL, Dosemeci M, Linet M, Zhang L, Xi L, Wacholder S, Lu W, Meyer KB, Titenko-Holland N, Stewart JT, Yin S, Ross D. 1997. Benzene poisoning, a risk factor for hematological malignancy, is associated with the NQO1 $^{609}C \rightarrow T$ mutation and rapid fractional excretion of chlorzoxazone. Cancer Res 57:2839–2842.

Roush GJ, Ott MG. 1977. A study of benzene exposure versus urinary phenol levels. Am Ind Hyg Assoc J 38:67–75.

Rupa DS, Hasegawa L, Eastmond DA. 1995. Detection of chromosomal breakage in the 1 cen-1q12 region of interphase human lymphocytes using multicolor fluorescence in situ hybridization with tandem DNA probes. Cancer Res 55:640–645.

Ruppert T, Scherer G, Tricker AR, Adlkofer F. 1997. *Trans,trans*-muconic acid as a biomarker of non-occupational environmental exposure to benzene. Int Arch Occup Environ Health 69:247–251.

Sato A, Nakajima T. 1979. Dose-dependent metabolic interaction between benzene and toluene in vivo and in vitro. Toxicol Appl Pharmacol 48:249–256.

Smith MT. 1996. The mechanism of benzene-induced leukemia: A hypothesis and speculations on the causes of leukemia. Environ Health Perspect 104(Suppl 6):1219–1225.

Smith MT, Yager JW, Steinmetz KL, Eastmond DA. 1989. Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. Environ Health Perspect 82:23–29.

Smith MT, Zhang L, Wang Y, Hayes RB, Li G, Wiemels J, Dosemeci M, Titenko-Holland N, Xi L, Kolachana P, Yin S, Rothman N. 1998. Increased translocations and aneusomy in chromosomes 8 and 21 among workers exposed to benzene. Cancer Res 58:2176–2181.

Snyder R, Hedli CC. 1996. An overview of benzene metabolism. Environ Health Perspect 104(Suppl 6):1165–1171.

Solomon E, Borrow J, Goddard AD. 1991. Chromosome aberrations and cancer. Science 254:1153–1160.

Sorsa M, Wilbourn J, Vainio H. 1992. Human cytogenetic damage as a predictor of cancer risk. IARC Sci Publ 116:543–554.

Tompa A, Major J, Jakab M. 1994. Monitoring of benzene-exposed workers for genotoxic effects of benzene: Improved-working-condition-related decrease in the frequencies of chromosome aberrations in peripheral blood lymphocytes. Mutat Res 304:159–165.

Tsai SP, Wen CP, Weiss NS, Wong O, McClellan WL, Gibson RL. 1983. Retrospective mortality and medical surveillance studies of workers in benzene areas of refineries. J Occup Med 25:685–692.

Turkel B, Egeli U. 1994. Analysis of chromosomal aberrations in shoe workers exposed long term to benzene. Occup Environ Med 51:50–53.

van Sittert NJ, Boogaard PJ, Beulink GDJ. 1993. Application of urinary *S*-phenylmercapturic acid test as a biomarker for low levels of exposure to benzene in industry. Br J Ind Med 50:460–469.

Waidyanatha S, Yeowell-O'Connell K, Rappaport SM. 1998. A new assay for albumin and hemoglobin adducts of 1,2- and 1,4- benzoquinones. Chem Biol Interact 115:117–39.

Wallace L. 1996 Environmental exposure to benzene: An update. Environ Health Perspect 104(Suppl 6):1129–1136.

Ward E, Hornung R, Morris J, Rinsky R, Wild D, Halperin W, Guthrie W. 1996. Risk of low red or white blood cell count related to estimated benzene exposure in a rubber-worker cohort (1940–1975). Am J Ind Med 29:247–257.

Witz G, Zhang Z, Goldstein BD. 1996. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. Environ Health Perspect 104(Suppl 6):1195–1199.

Yardley-Jones A, Anderson D, Lovell D, Jenkinson P. 1990. Analysis of chromosomal aberrations in workers exposed to low level benzene. Br J Ind Med 47:48–51.

Yeowell-O'Connell K, McDonald TA, Rappaport SM. 1996. Analysis of hemoglobin adducts of benzene oxide by gas chromatography-mass spectrometry. Anal Biochem 237:49–55.

Yeowell-O'Connell K, Rothman N, Smith MT, Hayes RB, Li G, Waidyanatha S, Dosemeci M, Zhang L, Yin S, Titenko-Holland N, Rappaport SM. 1998. Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. Carcinogenesis 19:1565–1571.

Yeowell-O'Connell K, Rothman N, Waidyanatha S, Smith MT, Hayes RB, Li G, Bechtold WE, Dosemeci M, Zhang L, Yin S, Rappaport SM. 2001. Protein adducts of 1,4-benzoquinone and benzene oxide among smokers and nonsmokers exposed to benzene in China. Cancer Epidemiol Biomarkers Prev 10:831–838.

Zhang L, Eastmond DA, Smith MT. 2002. The nature of chromosomal aberrations detected in humans exposed to benzene. Crit Rev Toxicol 32:1–42.

Zhang L, Rothman N, Wang Y, Hayes RB, Bechtold W, Venkatesh P, Yin S, Wang Y, Dosemeci M, Li G, Lu W, Smith MT. 1996. Interphase cytogenetics of workers exposed to benzene. Environ Health Perspect 104(Suppl 6):1325–1329.

Zhang L, Rothman N, Wang Y, Hayes RB, Li G, Dosemeci M, Yin S, Kolachana P, Titenko-Holland N, Smith MT. 1998 Increased aneusomy and long arm deletion of chromosomes 5 and 7 in the lymphocytes of Chinese workers exposed to benzene. Carcinogenesis 19:1955–1961.

Zhang L, Rothman N, Wang Y, Hayes RB, Yin S, Titenko-Holland N, Dosemeci M, Wang YZ, Kolachana P, Lu W, Xi L, Li GL, Smith MT. 1999. Benzene increases aneuploidy in the lymphocytes of exposed workers: A comparison of data obtained by fluorescence in situ hybridization in interphase and metaphase cells. Environ Mol Mutagen 34:260–268.

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