RESEARCH REPORT

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Biomarkers in Czech Workers Exposed to 1,3-Butadiene: A Transitional Epidemiologic Study

Richard J Albertini, Radim J Šrám, Pamela M Vacek, Jeremiah Lynch, Janice A Nicklas, Nico J van Sittert, Peter J Boogaard, Rogene F Henderson, James A Swenberg, Ad D Tates, Jonathan B Ward Jr, Michael Wright, and Others

Includes a Commentary by the Institute's Health Review Committee

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The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI supports research on all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 220 projects at institutions in North America and Europe and has published over 140 research reports.

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HEI STATEMENT
Synopsis of Research Report 116

Biomarkers in Butadiene-Exposed Workers

BACKGROUND

Butadiene is a four-carbon gaseous chemical synthesized for the manufacture of resins, plastics, and synthetic rubber. It is also produced by combustion; butadiene is present in cigarette smoke and emissions from motor vehicles and some stationary sources. The highest exposures, those that occur in occupational settings, may present a health concern because butadiene is known to be carcinogenic in rats and mice and some epidemiologic studies have implicated it as a human carcinogen by inhalation. Those studies have indicated that workers exposed to butadiene in rubber-producing factories also have an increased incidence of two types of cancer: cancers of the lymphatic system and cancers of the organs and systems of the body that produce blood cells. More recent and comprehensive studies of the same workers have indicated an increased risk of leukemia (but not other types of cancers) in workers with a long duration of employment in the rubber industry. On the basis of these epidemiologic studies, various government and international agencies have conducted risk assessments of butadiene's carcinogenicity and designated it as "potentially carcinogenic to humans," "a probable human carcinogen," and a "known human carcinogen."

In 1994, HEI initiated a research program to address the health risks of exposure to a series of chemicals, including butadiene, designated as toxic air pollutants by the US Environmental Protection Agency. In 1995, HEI issued Request for Qualifications 95-3, "Transitional Epidemiology Studies for Benzene or 1,3-Butadiene Biomarkers," which sought researchers with access to human populations exposed to either benzene or butadiene. The goal of RFQ 95-3 was to fund research to determine whether human exposure could be quantified by measuring the levels of certain biomarkers. Biomarkers are chemical compounds or physical characteristics that appear in bodily fluids or tissues after exposure to an exogenous agent. They can be specific indicators of exposure such as stable metabolites, metabolites bound to proteins or DNA, or genetic material that was altered because of the exposure.

Epidemiologic studies have encountered two primary difficulties in assessing exposure to carcinogenic agents. First, because the incidence of certain cancers is low, they have needed to study large populations to find an association between exposure and disease. Second, it is often difficult to accurately assess the level or time course of exposure to a possible cancer-causing agent in order to link past exposures to recent disease occurrences. In contrast with this, populations known to have been exposed to certain chemicals (such as groups of workers in a specific industry) show relatively high levels of biomarkers. Therefore, if biomarkers can accurately reflect the level or timing of exposure to a suspected carcinogen, they may be able to enhance exposure assessment in epidemiologic studies.

Dr Richard Albertini at the University of Vermont in Burlington organized groups of researchers from his own laboratory and laboratories in Galveston, Texas; Chapel Hill, North Carolina; Prague, Czech Republic; Amsterdam and Leiden, The Netherlands; and Sheffield, United Kingdom. Each group had expertise in identifying different biomarkers that appear after butadiene exposure. Dr Radim Šrám of the Laboratory of Genetic Ecotoxicology in Prague provided contact with butadiene-exposed workers in two production units of a factory near Prague.

APPROACH

The researchers proposed to determine whether biomarkers in the blood and urine of the exposed workers correlated with their personal exposure to butadiene. Šrám and coworkers in Prague collected blood and urine from male workers employed either in the butadiene monomer production plant or in the polymerization facility that used butadiene and styrene to produce rubber polymer. They also collected

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr Richard J Albertini at the University of Vermont, Burlington VT, with collaborators in seven European and US institutions. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

blood and urine from male administrative workers at the plant who had no direct occupational exposure to butadiene and served as control subjects. Each worker's personal exposure to butadiene in air was measured using a small air sampler attached to his clothes. Samplers were worn on several occasions over a 60-day period preceding and during the three days on which blood or urine samples were acquired. The air samplers were sent to a laboratory in Sheffield where butadiene levels were analyzed. For biomarker analyses, the Prague researchers sent portions of each blood and urine sample to the other research groups and kept a portion for their own use.

The study was conducted in a blinded fashion. Subject identities were known only to Albertini and the biostatistician for the study in Burlington, where codes were maintained, data were analyzed, and then matched to the three exposure groups.

The investigators focused their investigation on two types of biomarkers. The first type comprised *biomarkers of exposure* that were chosen specifically to indicate the level of butadiene in the body that resulted from an exposure. The researchers in Amsterdam and Chapel Hill determined the concentrations of two adducts that form when butadiene metabolites bind to hemoglobin in red blood cells. The Amsterdam researchers also measured levels of other exposure-related biomarkers, two metabolites that are excreted in urine when butadiene is detoxified in the body.

The second type of biomarker comprised *biomarkers of effect*, including gene mutations and structural changes in chromosomes. The researchers in Leiden and Galveston analyzed mutations in the *HPRT* gene by different methods and the Prague researchers determined the types and degrees of chromosomal changes.

In addition, the Prague and Burlington groups looked at factors that may affect *susceptibility* to carcinogens, such as changes in the genes that code for enzymes that metabolize butadiene. Such differences can modify an enzyme's activity and may affect an individual's response to possible butadiene-induced effects.

RESULTS AND INTERPRETATIONS

All the biomarkers of exposure were correlated with the measurements of butadiene recorded by the air samplers. Although the correlation between hemoglobin adducts and exposure levels was strongest, urinary metabolites were also found to be very useful measures of butadiene exposure.

No statistically significant correlations were found between any of the biomarkers of effect and butadiene exposure. Although these biomarkers were investigated, they were evaluated against exposure, not against health outcomes. Thus, no conclusions about health outcomes can be drawn from these results.

This very important and valuable study established the linkage between exposure to butadiene, as measured by comprehensive conventional sampling techniques, and several biological markers of such exposures. The integration of a comprehensive exposure assessment with a series of logical biomarker analyses was an outstanding feature of this complex international study. Of the many biomarkers analyzed, the biomarkers of exposure (particularly hemoglobin adducts) may prove to be valuable in future epidemiologic studies of the health effects of butadiene exposure.

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CONTENTS

Research Report 116

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Richard J Albertini, Radim J Šrám, Pamela M Vacek, Jeremiah Lynch, Janice A Nicklas, Nico J van Sittert, Peter J Boogaard, Rogene F Henderson, James A Swenberg, Ad D Tates, Jonathan B Ward Jr, Michael Wright

with Marinel M Ammenheuser, Blanka Binkova, Walter Blackwell, Franz A de Zwart, Dean Krako, Jennifer Krone, Hendricus Megens, Petra Musilová, Gabriela Rajská, Asoka Ranasinghe, Judah I Rosenblatt, Pavel Rössner, Jiri Rubeš, Linda Sullivan, Patricia Upton, and Ailko H Zwinderman

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HEI STATEMENT

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

INVESTIGATORS' REPORT

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

Abstract 1
Overview 2
Epidemiologic Studies in Humans 3
Cancer Studies in Rodents 4
BD Metabolism 4
BD Metabolism in Rodents 6
Molecular Dosimetry 7
Genotoxicity of BD 8
Molecular Epidemiologic Studies in Humans 9
The Current Study 11
Specific Aims 12
Phase I 12
Phase II 12
Supplemental Analyses 13
Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods 14
Subject Selection and Exposure Assessment 14
Procedure to Assure Blinded Analyses 15
Chain of Custody 15
Sample Acquisition and Handling 15
Subjects' Biological Samples 15
Urine 15
Blood 15
Isolation of MNCs and Plasma 16
Isolation of Erythrocytes 16
Sorbent Tubes for BD and VOCs 16
Data Processing and Statistical Analysis 16
Data Processing 16
Statistical Analysis 17
Workers' Exposure Groups, Personal Characteristics, and Metabolic Genotypes . . . 18
Exposure Groups 18
Workplace Environment 18
Results: Job and Task Analysis 18
Personal Characteristics 19

Continued

Questionnaires 19
Results: Characteristics and Coexposures 21
Metabolic Genotypes 23
Genotyping Methods 25
GSTM1 and *GSTT1* 25
ADH2, *ADH3*, *EH*, and *CYP2E1* 25
Results: Genotypes 26
Measurement of Occupational Exposure to BD 28
Exposure Assessment Protocol 28
Exposure Measurements from Personal Monitors 28
Workplace Area Measurements 29
Exposure Sampling and Analysis 29
BD: Materials and Methods 29
VOCs: Materials and Methods 31
Quality Control 31
Statistical Methods 32
Results 32
BD Exposure Measurements from Personal Monitors 32
Summary Metrics for BD Exposure from Personal Monitors 34
Workplace Area Measurements of BD 38
Concurrent VOC Exposure Measurements from Personal Monitors 40
Workplace Area Measurements of VOCs .. 41
Discussion 42
Biomarkers of Exposure: BD Metabolites in Urine 43
Metabolism of BD to Form Urinary M2 and M1 43
M2 and M1 as Biomarkers of Exposure 43
Methods 44
Synthesis of Reference and Internal Standards 45
Calibration Standards and Curves 45
QC Urine Standards 45
QC Procedures 45
Analysis of Urinary M2 and M1 45
Statistical Methods 46
Results 46
Measurement of Urinary M2 and M1 46
Correlations Among Urinary Biomarkers and Personal Exposure Monitoring Results 47
Influence of Genotypes and Metabolic Pathways (GSH Conjugation vs EH Hydrolysis) 49
Discussion 50
Biomarkers of Exposure: Hb Adducts 53
Materials and Methods for HBVal Analysis ... 56
Synthesis of Reference and Internal Standards 56
Calibration Standards and Curves 57
QC Procedures 57
HBVal Analysis 57
Materials and Methods for THBVal Analysis 57
Synthesis of Internal Standards 57
Calibration Standards and Curves 57
QC Standards 57
QC Procedures 58
THBVal Analysis 58
Statistical Analyses 58
Results 58
HBVal Adduct Concentrations 58
HBVal Adducts and Genotypes 60
THBVal Adduct Concentrations 60
THBVal Adducts and Genotypes 60
Correlation Between HBVal and THBVal .. 61
Ratio of THBVal to HBVal 61
Discussion 61
Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes 63
Materials and Methods 65
Cloning Assay 65
Receipt and Handling of Samples 65
Quality Control Procedures 65
Thawing Frozen Samples 65
Preincubation 65
Culture Medium 67
Cloning Lymphocytes 67
Scoring Clones and Calculating CEs and MFs 67
Autoradiographic Assay 67
Receipt and Handling of Samples 67
Quality Control Procedures 68
Thawing Frozen Samples 68
Culture Medium 68
Incubating Samples for *HPRT* Variant Selection 68
Fixing Cells and Preparing Slides 68
Scoring Slides and Calculating VFs 68
Duplicate Scoring 68
Statistical Analysis of Cloning and Autoradiographic Assay Data 69
Results of the Cloning Assay 69
MFs 69
Differences in MFs Among the Exposure Groups 70
Association Between MF and Exposure to BD 70
Effect of Smoking on MFs 70

Continued

Effect of Age on MFs 71
Effect of Genotype on MFs 71
Correlations of MFs with Urinary Metabolites or Hb Adducts 71
CEs 71
Differences in CEs Among the Exposure Groups 72
Association Between CE and Exposure to BD 72
Numbers of Clonable Cells 72
Numbers of Living Cells 72
Results of the Autoradiographic Assay 72
Quality of the Lymphocyte Samples 72
Results for VF by Exposure Group 73
ECs 75
Consistency of Slide Scoring 75
Comparison of Autoradiographic and Cloning Assays 76
Discussion 76
The Cloning Assay 76
The Autoradiographic Assay 78
Sample Quality 78
Relation of *HPRT* VF to BD Exposure . . 79
Factors That Could Affect the Results of *HPRT* Assays 81
Comparison of the Cloning and Autoradiographic Assays 82
Conclusion 82
Biomarkers of Effect: *HPRT* Mutational Spectra 83
Methods 83
Expansion of Clones 83
PCR Amplification and DNA Sequencing 86
Statistical Analysis 87
Results 87
Discussion 93
Biomarkers of Effect: Cytogenetic Analysis . . . 97
Methods 98
Chromosomal Aberrations Identified by the Conventional Method 98
Chromosomal Changes Identified by FISH 98
SCEs 99
Statistical Methods 99
Results 99
Chromosomal Aberrations Identified by the Conventional Method 99
Chromosomal Changes Identified by FISH 104
SCEs 106
Discussion and Conclusions 107
Correlations Among Biomarkers 108
Correlations Among Biomarkers of Exposure 108
Correlations Among Biomarkers of Exposure and Biomarkers of Effect 109
HPRT Mutations Assessed by the Cloning Assay (Mutant Frequencies) 109
HPRT Mutations Assessed by the Autoradiographic Assay (Variant Frequencies) 109
Cytogenetic Changes 110
Chromosomal Aberrations Identified by the Conventional Method 110
Chromosomal Changes Identified by FISH 110
SCEs 110
Correlations Among the Biomarkers of Effect: *HPRT* Mutations and Cytogenetic Endpoints 111
Conclusions 111
Discussion and Conclusions 112
Exposure Assessment 112
Study Subjects 113
Urinary Metabolites 113
Hemoglobin Adducts 115
Genotoxicity 116
Conclusion 118
Acknowledgments 119
References 119
Appendix A. HEI Quality Assurance Report . . 135
About the Primary Authors 137
Collaborating Institutions 138
Other Publications Resulting from This Research 139
Abbreviations and Other Terms 139
Alternate Terms for Butadiene and Its Metabolites and Adducts 141

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.

Introduction 143
Scientific Background 144
What Is a Transitional Epidemiologic Study? 145
Biomarkers and Molecular Epidemiology . . 145
Biomarkers Chosen for This Study 145
Biomarkers of Exposure 146
Urinary Metabolites 146
Hb Adducts 146

Continued

Biomarkers of Effect 147
HPRT Mutations 147
Mutational Spectra 148
SCEs and Chromosomal Aberrations .. 148
Factors That May Affect Susceptibility ... 148
Genetic Polymorphisms and Cytogenetic Damage 149
Technical Evaluation 149
Study Design 149
Methods 151
Exposure Assessment 151
Biomarkers of Exposure 152
Urinary Metabolites 152
Hb Adducts: HBVal and THBVal 152
Biomarkers of Effect 152
HPRT Mutants and Variants 152
HPRT Mutational Spectra 152
Cytogenetic Changes: SCEs and Chromosomal Aberrations 152
Factors That May Affect Susceptibility: Metabolic Genotypes 153
Statistical Analyses 153
Results and Interpretations 153
Exposure Assessment 153
Biomarkers of Exposure 154
Urinary Metabolites 154
Hb Adducts 155
Biomarkers of Effect 155
HPRT Mutant Cells Assessed via the Cloning Assay 155
HPRT Variant Cells Assessed via the Autoradiographic Assay 156
Mutational Spectra 156
Cytogenetic Analyses 156
Factors That May Affect Susceptibility ... 157
Correlations Among Biomarkers 157
Correlations Among the Biomarkers of Exposure 157
Correlations Among the Biomarkers of Exposure and the Biomarkers of Effect 157
Correlations Among the Biomarkers of Effect 157
Discussion of Mutation Findings 157
Summary 158
Acknowledgments 159
References 160

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INVESTIGATORS' REPORT

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ABSTRACT

A multiinstitutional, transitional epidemiologic study was conducted with a worker population in the Czech Republic to evaluate the utility of a continuum of nondisease biological responses as biomarkers of exposure to 1,3-butadiene (BD)* in an industrial setting. The study site included two BD facilities in the Czech Republic. Institutions that collaborated in the study were the University of Vermont (Burlington, Vermont, USA); the Laboratory of Genetic Ecotoxicology (Prague, the Czech Republic); Shell International Chemicals, BV (Amsterdam, The Netherlands); the University of North Carolina at Chapel Hill (Chapel Hill, North Carolina, USA); University of Texas Medical Branch at Galveston (Galveston, Texas, USA); Leiden University (Leiden, The Netherlands); and the Health and Safety Laboratory (Sheffield, United Kingdom).

Male volunteer workers (83) participated in the study: 24 were engaged in BD monomer production, 34 in polymerization activities, and 25 plant administrative workers served as unexposed control subjects.

The BD concentrations experienced by each exposed worker were measured by personal monitor on approximately ten separate occasions for 8-hour workshifts over a 60-day exposure assessment period before biological samples were collected. Coexposures to styrene, benzene, and toluene were also measured. The administrative control workers were considered to be a homogeneous, unexposed group for whom a series of 28 random BD measurements were taken during the exposure assessment period. Questionnaires were administered in Czech to all participants.

At the end of the exposure assessment period, blood and urine samples were collected at the plant; samples were fractionated, cryopreserved, and kept frozen in Prague until they were shipped to the appropriate laboratories for specific biomarker analysis. The following biomarkers were analyzed:

- polymorphisms in genes involved in BD metabolism (Prague and Burlington);
- urinary concentrations of 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene and 2-hydroxy-1-(*N*-acetylcysteinyl)-3-butene (M2 [refers to an isomeric mixture of both forms]) (Amsterdam);
- urinary concentrations of 1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane (M1) (Amsterdam);
- concentrations of the hemoglobin (Hb) adducts *N*-(1-[hydroxymethyl]-2-propenyl)valine and *N*-(2-hydroxy-3-butenyl)valine (HBVal [refers to an isomeric mixture of both forms]) (Amsterdam);
- concentrations of the Hb adduct *N*-(2,3,4-trihydroxybutyl)valine (THBVal) (Chapel Hill);
- T cell mutations in the *hypoxanthine phosphoribosyltransferase* (*HPRT*) gene (autoradiographic assay in Galveston with slide review in Burlington; cloning assay in Leiden with mutational spectra determined in Burlington); and
- chromosomal aberrations by the conventional method and by fluorescence in situ hybridization [FISH]), and cytogenetic changes (sister chromatid exchanges [SCEs] (Prague).

*A list of abbreviations and other terms appears at the end of the Investigators' Report. That list includes other names by which butadiene and its metabolites and adducts might be known.

This Investigators' Report is one part of Health Effects Institute Research Report 116, 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 Richard J Albertini, Genetic Toxicology Laboratory, University of Vermont, 655 Spear Street, Building C, Burlington VT 05405; *Richard.Albertini@uvm.edu*.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R82811201 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

All assay analysts were blinded to worker and sample identity and remained so until all work in that laboratory had been completed and reported. Assay results were sent to the Biometry Facility in Burlington for statistical analyses.

Analysis of questionnaire data revealed that the three exposure groups were balanced with respect to age and years of residence in the district, but the control group had significantly more education than the other two groups and included fewer smokers.

Group average BD exposures were 0.023 mg/m^3 (0.010 ppm) for the control group, 0.642 mg/m^3 (0.290 ppm) for the monomer group, and 1.794 mg/m^3 (0.812 ppm) for the polymer group; exposure levels showed considerable variability between and within individuals. Styrene exposures were significantly higher in the polymer group than in the other two groups.

We found no statistically significant differences in the distributions of metabolic genotypes over the three exposure groups; genotype frequencies were consistent with those previously reported for this ethnic and national population. Although some specific genotypes were associated with quantitative differences in urinary metabolite concentrations or Hb adduct dose-response characteristics, none indicated a heightened susceptibility to BD.

Concentrations of both the M2 and M1 urinary metabolites and both the HBVal and THBVal Hb adducts were significantly correlated with group and individual mean BD exposure levels; the Hb adducts were more strongly correlated than the urinary metabolites. By contrast, no significant relations were observed between BD exposures and *HPRT* gene mutations (whether determined by the autoradiographic or the cloning method) or any of the cytogenetic biomarkers (whether determined by the conventional method or FISH analysis). Neither the mutational nor the cytogenetic responses showed any association with genotypes. The molecular spectrum of *HPRT* mutations in BD-exposed workers showed a high frequency of deletions; but the same result was found in the unexposed control subjects, which suggests that these were not due to BD exposure. This lack of association between BD exposures and genetic effects persisted even when control subjects were excluded from the analyses or when we conducted regression analyses of individual workers exposed to different levels of BD.

OVERVIEW

BD is a highly volatile, four-carbon chemical (C_4H_6; CAS 106-99-0). It is colorless and mildly aromatic, has a molecular weight of 54.09, and the chemical structure of CH_2=CH–CH=CH_2 (Weast 1989). With a boiling point of −4.4°C, a vapor pressure of 1900 mm Hg at 20°C, and low water solubility, BD exists naturally as a gas and partitions almost entirely to the atmosphere on release (Kirshenbaum 1978). Once released, atmospheric destruction of BD occurs in several ways, including reactions with ozone, nitrate radicals, or photochemically produced hydroxyl radicals. BD spontaneously forms the 4-vinyl-cyclohexane dimer, is flammable, and can form explosive peroxides at the appropriate atmospheric concentrations (Kirshenbaum 1978; US Department of Health and Human Services [DHHS] 1992).

BD is an important industrial chemical used as an intermediate in the production of polymers, elastomers, and other chemicals. Although its major uses are in the manufacture of styrene-BD rubber (SBR) and thermoplastic resins, it is present in many products including the fungicides captan and captfol (US Environmental Protection Agency [EPA] 1994).

Worldwide, the annual industrial production of BD is approximately 12 billion pounds, of which 3 billion are produced in the United States alone (Morrow 1990; US International Trade Commission 1990). Industrial workers are exposed to the highest BD levels; 8-hour time-weighted average (TWA) levels between 10 and 370 ppm (22.1 to 817.7 mg/m^3) have been reported (reviewed in Melnick and Kohn 1995; European Centre for Ecotoxicity and Toxicology of Chemicals 1997; Himmelstein et al 1997; International Agency for Research on Cancer [IARC] 1999). These exposures and, to a lesser extent, those in the vicinity of industrial plants that produce or use the chemical are of most immediate concern because of the high concentrations to which workers may be exposed.

Despite its importance as an industrial chemical, the major source of BD in the ambient environment is emissions from gasoline- and diesel-powered vehicle engines. BD is formed as a product of incomplete combustion of fossil fuels and is also released from burning tobacco, biomass, and automobile tires. More than 98% of the BD released to the environment is from mobile and other miscellaneous combustion sources (EPA 1994). Section 112 of the Clean Air Act Amendments of 1990 (US Congress 1991) lists BD as 1 of 188 chemicals designated as hazardous; Section 211 of the Amendments lists it as 1 of 5 chemicals designated as mobile-source toxic air pollutants. Furthermore, the EPA includes BD in its lists of

hazardous air pollutants and mobile-source air toxics (EPA 1999, 2001). The EPA is required to develop a national strategy to control the emissions of these hazardous air pollutants and reduce the cancer risk attributable to them.

A population much larger than industrial workers is exposed, albeit at lower individual levels, to 98% of the total BD released into the atmosphere from nonindustrial sources; these concentrations are estimated to be in the range of less than 2.21 to 22 $\mu g/m^3$ (< 1 to 10 parts per billion [ppb]), with the highest levels found in urban air. Exposures at these levels may also have potential health consequences. Nonindustrial indoor exposures may exceed outdoor exposures, especially when tobacco smoke is present. Measured BD levels in smoke-filled bars have ranged from 2.7 to 19 $\mu g/m^3$ (1.2 to 8.6 ppb) (Löfroth et al 1989; Brunnemann et al 1990). The high volatility and poor water solubility of BD ensures that inhalation is the major route of human exposure.

The potential for widespread human exposure to BD has focused attention on its health effects. Initially, BD was thought to be relatively nontoxic; early control measures were directed at the irritating effects that result from short-term exposures. On this basis, the permissible occupational exposure limit (OEL) was set at 2210 mg/m^3 (1000 ppm) (US Occupational Safety and Health Administration [OSHA] 1990). This standard did not take into consideration, however, that by the mid-1980s, long-term animal studies of BD inhalation had revealed carcinogenicity in rodents (Huff et al 1985; Owens et al 1987; Irons et al 1989). At the present time, reproductive and heritable disorders and cancer are the major human health concerns related to BD exposure.

Several risk assessments of BD carcinogenicity have been conducted by US and other health agencies over the past 15 years. The California Air Resources Board (CARB; 1991), US National Institute for Occupational Safety and Health (NIOSH) (DHHS 1991), and OSHA (1996) have evaluated BD for its potential carcinogenicity; CARB provided no formal classification, but NIOSH and OSHA both concluded that it is at least potentially carcinogenic to humans. As early as 1985, the EPA classified BD as a probable human carcinogen (EPA 1985). The most recent Cancer Risk Assessment by EPA (2002) has concluded that BD is carcinogenic to humans exposed by inhalation, with a lifetime exposure of 0.01 ppb producing an increase of one cancer per million exposed individuals. This determination of risk was based on the human data discussed above. The US National Toxicology Program (NTP) named BD as a known human carcinogen in the 9th edition of its *Report on Carcinogens* (DHHS 2000). The Canadian Environmental Protection Act (Environment Canada 1999) proposed a classification of known human carcinogen. IARC designated BD as a probable human carcinogen (Class 2A) in 1992; this classification was reviewed in 1999 and, although upgrading BD to known human carcinogen (Class 1) had much support, the final decision was to retain Class 2A until more definitive human data are available (IARC 1999).

Both great concerns and considerable uncertainties exist about the human cancer risks resulting from BD exposure. The uncertainties are due to (1) marked species differences between rats and mice in their cancer susceptibility that make extrapolating study results to humans problematic; (2) inconsistencies in human epidemiologic data that allow for varied interpretations; and (3) the limited information about levels of human exposures.

In the following sections, we summarize briefly the data relevant to BD's possible carcinogenicity and reproductive toxicity for humans (much of which is based on studies in animals) and the gaps in knowledge that limit toxicologic evaluation of this agent. We present the potential for molecular epidemiologic studies to fill these gaps and the need for transitional epidemiologic studies, which are the bridge between laboratory and field studies, to properly interpret applied molecular studies.

EPIDEMIOLOGIC STUDIES IN HUMANS

Studies have been reported both for workers having relatively pure BD exposures (ie, in BD monomer production) and for workers with more complex exposures (ie, engaged in BD polymerization such as in the SBR industry). The largest study of monomer production workers involved 2795 persons employed for at least 6 months between 1952 and 1994 (Divine 1990; Divine et al 1993; Divine and Hartman 1996). Two smaller studies of monomer production workers were also conducted (Downs et al 1987; Ward et al 1995, 1996a; Cowles et al 1994). Although one of the small studies and earlier updates of the large-cohort study reported increases in hematologic malignancies (mainly lymphomas), these findings were inconsistent. Overall, the evidence for cancer induction in monomer production workers has been weak and without dose-related responses. Positive associations between BD exposure and malignancies in monomer production workers were reported only for workers employed before 1950.

Different results have been found in studies of BD polymerization workers. The largest SBR industry study of 15,649 men from eight North American SBR plants, which has been reported in whole or in part in several publications, found an increased leukemia risk (Meinhardt et al 1982; Matanoski et al 1982, 1993; Santos-Burgoa et al 1992;

Delzell et al 1996a,b; Macaluso et al 1996, 1997). However, that study found no increases in other lymphohematopoietic malignancies. The excess leukemia deaths were in job categories with relatively high BD exposures. Retrospective dose reconstructions suggested an exposure-response relation for BD, but no relation for styrene (Macaluso et al 1996, 1997).

The epidemiologic findings in BD-exposed workers suggest but do not prove BD's carcinogenicity for humans. Difficulties in interpreting these findings result from lack of concurrent information on BD exposures, potentially confounding factors, and the discordance between results in the monomer production and SBR workers. Because these epidemiologic studies have included almost the entire North American BD worker population, it is unlikely that additional data on direct human carcinogenicity will be forthcoming in the near future.

CANCER STUDIES IN RODENTS

The different carcinogenicity classifications of BD are based in part on studies of mice and rats. Three of these were long-term bioassay studies with mice: In the first study, mice were exposed to 625 or 1250 ppm (1381.3 or 2762.5 mg/m^3) BD; but the study was terminated early because of excess mortality due to lymphomas (Huff et al 1985). In the second study, mice were exposed to 6.25, 20, 62.5, 200, or 625 ppm (13.8, 44.2, 138.1, 442.0, or 1381.3 mg/m^3) BD and showed significantly increased incidences of tumors at multiple organ sites in both sexes down to 20 ppm and of lung tumors in females down to 6.25 ppm (Melnick et al 1990; DHHS 1993). A third study compared tumor incidences in B6C3F$_1$ mice and US National Institutes of Health Swiss mice; this study demonstrated that BD-induced lymphomas were not solely attributable to retroviral activation in the B6C3F$_1$ mice (Irons et al 1989). In the single cancer bioassay study with rats, Sprague-Dawley rats were exposed to 1000 or 8000 ppm (2210 or 17,680 mg/m^3) BD (Owen et al 1987). This study also showed different neoplasms at multiple organ sites; however, the rats were two to three orders of magnitude less sensitive to BD cancer induction than mice. Differential sensitivity between the two species has been a major source of uncertainty in extrapolating animal data to humans.

In addition to the standard, intact-animal cancer bioassays, other toxicologic evaluations have indicated that BD and some of its metabolites are carcinogenic. More than thirty years ago, studies showed skin cancers in mice induced by local applications of the metabolites 1,2-epoxy-3-butene (BDO) and 1,2,3,4-diepoxybutane (BDO_2) (Van Duuren et al 1963, 1965). Early research also showed that (1) DL-isomeric mixtures of BDO_2 (made up of equal quantities of isomers that are structural mirror images of each other) induced local sarcomas in both rats and mice after subcutaneous injections, and (2) the L form was carcinogenic to mice after intraperitoneal injections (Van Duuren et al 1966). BDO_2 administered to Sprague-Dawley rats by injection produced elevated levels of guanylate cyclase (which was considered to be a surrogate marker for cancer) in liver, kidney, bladder, stomach, pancreas, colon, heart, and lung; this suggested that BDO_2 could produce malignancies (Vesely and Levey 1978). Finally, rat embryo fibroblasts treated in vitro with BDO_2 showed features of structural transformation; injecting these cells into neonatal rats resulted in malignant tumors (Wolman and Sivak 1975).

Certain mutations (eg, K-*ras* and H-*ras*) and rearrangements of genomic regions associated with tumor suppressor genes have been observed in the BD-induced tumors of mice, which implicates genetic changes as the carcinogenic mechanism (Goodrow et al 1990, 1994). Although genetic change is a mechanism applicable to all species (including humans), the chemical metabolites actually responsible for carcinogenicity must be produced in the species for cancer to occur. Therefore, one explanation for the differential susceptibility to BD's carcinogenicity between mice and rats—with relevance for human risk assessment—is differential metabolism of BD in the two rodent species.

BD METABOLISM

BD itself is not biologically active. It must be metabolized in vivo to produce carcinogenic products. An extremely complex metabolic pathway common to all species has been identified for BD; a simplified version is depicted in Figure 1 (modified from Himmelstein et al 1997). All species oxidize BD to intermediates that can react with DNA and are therefore potentially carcinogenic; all species can also detoxify these intermediates by converting reactive regions to less reactive sites and, in the process, making the compounds more water-soluble for excretion in the urine.

BD is initially oxidized to BDO, a reaction mediated primarily by cytochrome P450 2E1 (CYP2E1) (reviewed in Csanády et al 1992; Dueschere and Elfarra 1994; Himmelstein et al 1997). Further oxidation of BDO produces BDO_2 (Seaton et al 1995). BDO can also be detoxified by conjugation with GSH (mediated by glutathione *S*–transferase [GST]) or by hydrolysis (mediated by epoxide hydrolase [EH]); the latter produces 1,2-dihydroxy-3-butene (BD-diol) (reviewed in Himmelstein et al 1997). Both BDO_2 and

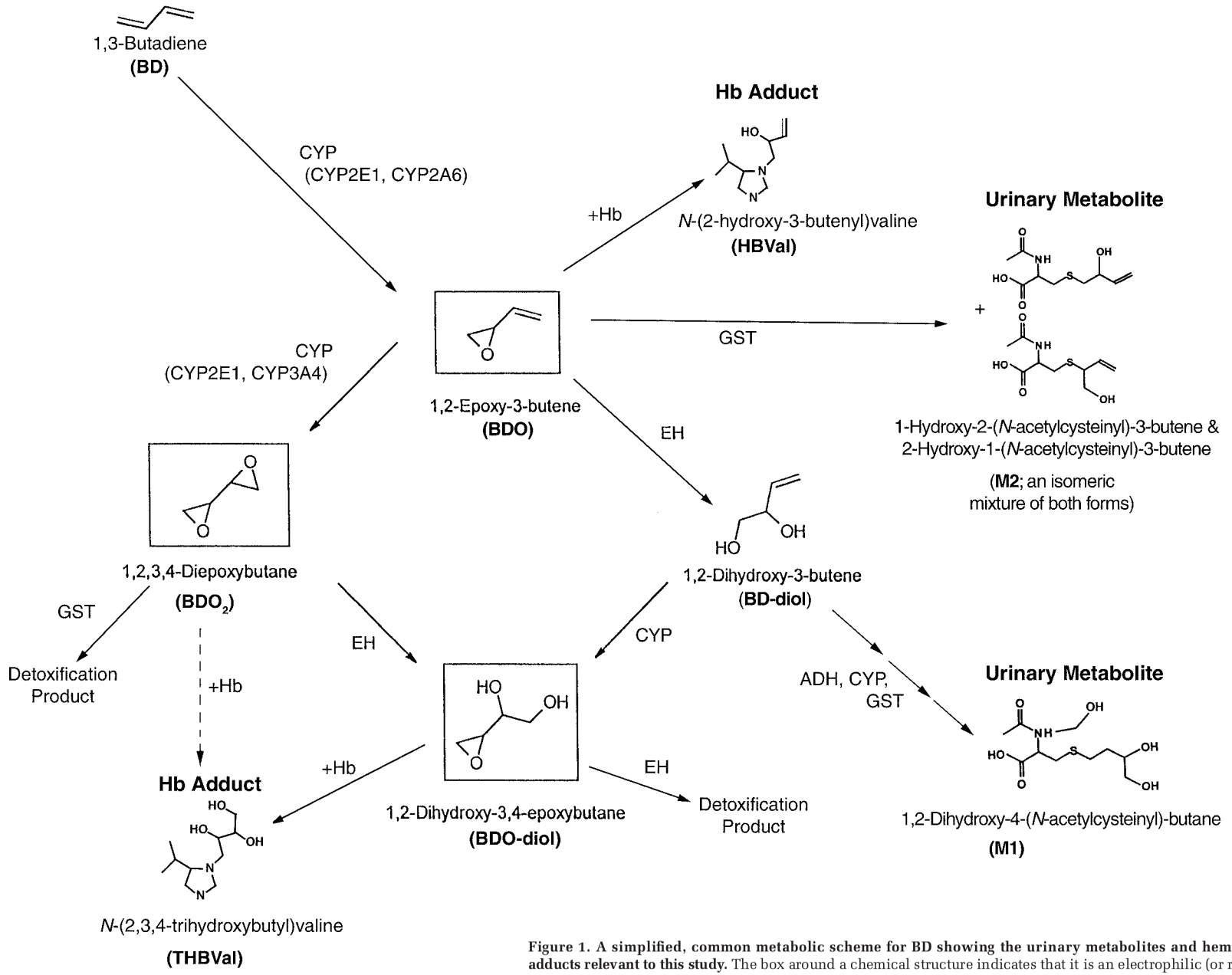

Figure 1. A simplified, common metabolic scheme for BD showing the urinary metabolites and hemoglobin adducts relevant to this study. The box around a chemical structure indicates that it is an electrophilic (or reactive) epoxide metabolite; arrow with broken lines indicate some uncertainty about this pathway.

BD-diol undergo further conversions, BDO_2 by EH-mediated hydrolysis and BD-diol presumably by oxidation mediated by cytochrome P450–related enzymes (CYP), to produce 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). BDO, BDO_2, and BDO-diol are reactive electrophilic compounds; that is, they react with nucleophilic centers in macromolecules (such as proteins and nucleic acids). Such reactions with nucleic acids can result in direct damage to DNA. Thus, these three compounds, possibly among others, are the potential carcinogenic intermediates of BD metabolism in vivo.

The BD metabolism scheme depicted in Figure 1 is complicated by the fact that the three electrophilic intermediates may exist in more than one stereoisomeric form (Krause and Elfarra 1997; Nieusma et al 1997, 1998; reviewed in HEI 2000). Stereoisomers are different three-dimensional forms of a compound that have identical chemical composition but that arise because of atoms bonding asymmetrically to a carbon atom (Figure 2). For example, BDO has one such asymmetric center that allows for two stereoisomeric forms, referred to as the left-hand (*S*) and right-hand (*R*) isomers. BDO_2 has two asymmetric centers that allow for three stereoisomeric forms: the (*S*,*S*) and (*R*,*R*) forms, in which the asymmetric centers are both in the *S* or *R* configuration, and a third *meso* form, in which one center has the (*S*) and the other center has the (*R*) configuration to produce overall spatial symmetry in the molecule. An isomeric mixture of BDO_2 (also referred to as $\pm BDO_2$) contains both the *S*,*S* and *R*,*R* forms. Hydration of these stereoisomeric forms of BDO_2 can result in four stereoisomers of BDO-diol: the (2*S*,3*R*), (2*R*,3*S*), (2*R*,3*R*), and (2*S*,3*S*) forms. Therefore, the metabolism of BD according to the scheme shown in Figure 1 can yield nine different stereoisomeric forms of the three electrophilic intermediates (Figure 2). Because enzyme reactions with an intermediate may be specific to a particular stereoisomer, its level may dictate the final metabolic outcomes in vivo.

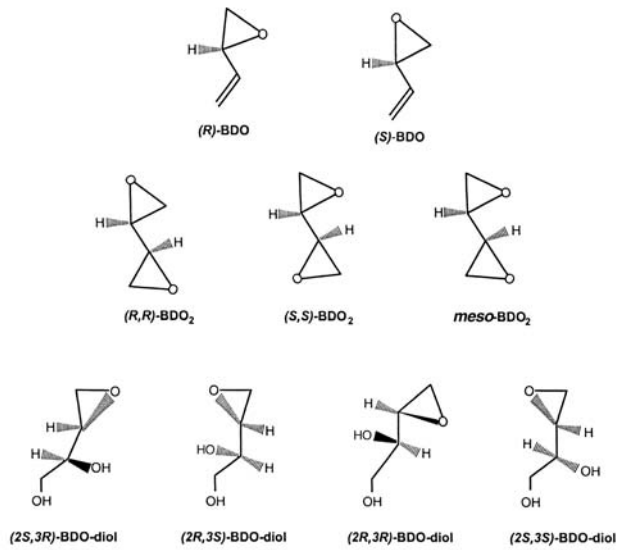

Figure 2. Stereochemistry of BD. In some molecules, the asymmetric bonding of some atoms to a carbon atom causes "left hand" (*S*) and "right hand" (*R*) forms (called stereoisomers) to exist. These forms with asymmetric centers, which are considered mirror images, are chemically similar but differ in their three-dimensional configuration. Oxidation of BD to BDO produces one asymmetric center, hence (*S*)- and (*R*)-BDO. Further oxidation converts (*R*)- and (*S*)-BDO to three forms of BDO_2. [The designations (*R*,*R*) and (*S*,*S*) indicate two asymmetric centers; the *meso* form also has two asymmetric centers, but their spatial configurations yield overall symmetry within the molecule.] Hydration of the three BDO_2 forms can produce four forms of BDO-diol. Thus, nine possible stereochemical configurations exist for the three BD epoxides. (Adapted with permission from Nieusma et al 1998.)

Figure 1 also depicts the pathways that lead to the urinary M2 and M1 metabolites. First, direct GST-mediated conjugation of BDO with glutathione (GSH) leads to the detoxification product M2, which is a biomarker of the conjugation detoxification pathway. Second, further metabolism of BD-diol by multiple steps involving alcohol dehydrogenase and aldehyde dehydrogenase (ADH), CYP, and GST-mediated conjugation with GSH leads to the production of M1. M1 is a biomarker of the hydrolytic detoxification pathway for BDO (via BD-diol) because it is mediated initially by EH. The ratio of M1/(M1 + M2) in urine defines the relative importance of hydrolysis vs conjugation in the detoxification of BDO (Bechtold et al 1994; reviewed in Henderson et al 1996).

BD METABOLISM IN RODENTS

In vitro studies have shown that mice are more efficient than rats in oxidizing BD to BDO (Csanády et al 1992). The second oxidation step from BDO to BDO_2 also has been shown in vitro; this reaction occurs more readily in mouse liver microsomes than in rat or human liver microsomes (Csanády et al 1992; Seaton et al 1995; Krause and Elfarra 1997).

In vivo studies of BD metabolism in mice and rats have also shown large interspecies differences. Mice exposed by inhalation removed BD from the atmosphere twice as rapidly as rats but retained fivefold more radioactivity from inhaled [^{14}C]BD (Bond et al 1986; Kreiling et al 1986; Dahl et al 1991; Richardson et al 1999). Mean concentrations of BDO in liver and lung following BD exposure by inhalation were approximately 10-fold to 15-fold greater in mice than in rats (Himmelstein et al 1995). Thornton-Manning and colleagues (1995) found both BDO and BDO_2 in mice and rats exposed to BD by inhalation; concentrations of both metabolites were greater in mice than in rats: BDO

was threefold to eightfold greater, and BDO_2 was 40-fold to 160-fold greater. M1/(M1 + M2) ratios in urine for mice (0.19) and rats (0.52) exposed to BD by inhalation indicate that detoxification by GSH conjugation predominates in mice, but that hydrolysis is more important in rats (Henderson et al 1996).

Thus, mice are more efficient in oxidizing BD to electrophilic metabolites (especially to BDO_2), whereas rats are more efficient in hydrolytic detoxification. This may be the major reason why BD is more carcinogenic in mice than in rats. It is of extreme importance, therefore, that the human metabolic profile of this agent be determined because it may indicate which, if either, of the rodent species is the appropriate cancer model for risk extrapolation.

MOLECULAR DOSIMETRY

Chemical carcinogens that act through damage to DNA are referred to as electrophilic; that is, they react with nucleophilic centers in macromolecules such as proteins and nucleic acids. This electrophilic property is found in the BD metabolites. Some chemicals that are bound covalently to proteins neither damage the genetic material nor lead directly to cancer. However, the resulting adducts can be measured to provide an indication of the internal dose of the in vivo concentrations of different metabolites of a chemical to which a subject has been exposed. The BD metabolite BDO has been shown to react with Hb to form HBVal adducts (Osterman Golkar et al 1991, 1993; Van Sittert and Van Vliet 1994); THBVal, a second Hb adduct, has also been identified (Perez et al 1997; Swenberg et al 2000a). These two adducts are particularly useful as internal dosimeters of BD metabolites (Osterman-Golkar et al 1993; Perez et al 1997; Swenberg et al 2000a).

Osterman-Golkar and associates (1991, 1993) measured the HBVal Hb adducts produced by BDO in both $B6C3F_1$ mice and Sprague-Dawley rats exposed to BD by inhalation; they found linearity in the mouse and nonlinearity (supralinearity) in the rat; that is, at 10 to 100 ppm BD (22.1 to 221 mg/m^3), mice showed a fivefold greater adduct concentration than rats, whereas at exposures of less than 10 ppm, the concentration in mice was only 1.7-fold higher. Swenberg and coworkers (2000a) showed that mice exposed to higher levels of BD showed 2.3-fold (males) and 1.9-fold (females) greater HBVal adduct concentrations than did rats.

The THBVal Hb adducts can be produced by either BDO_2 or BDO-diol, although current evidence indicates that they derive almost entirely from BDO-diol (Perez et al 1997; Koivisto et al 1999). After exposure to 1000 ppm (2210.0 mg/m^3) BD by inhalation, the average concentrations of THBVal adducts in males and females were 4.3-fold greater in mice than in rats (Swenberg et al 2000a). Within a species, the ratio of total THBVal to total HBVal adduct concentrations was 6.3 for male mice, 3.4 for female mice, 1.8 for male rats, and 2.5 for female rats; this indicates that the BDO-diol metabolite is the most abundant electrophilic product of in vivo BD metabolism in both species and that mice produce more than rats (Perez et al 1997; Koivisto et al 1999; Swenberg et al 2000a).

BD metabolites also form covalent adducts with DNA in all species (Citti et al 1984; Jelitto et al 1989; Leuratti et al 1994; Neagu et al 1995; Bolt and Jelitto 1996; Kumar et al 1996; Selzer and Elfarra 1996a,b; Koivisto et al 1996, 1997, 1998a,b, 1999; Tretyakova et al 1997a,b,c, 1998; Boogaard et al 1998, 2000, 2001, unpublished results 2003; Koc et al 1999; Blair et al 2000). N7-(2-hydroxy-3-butenyl)guanine or N6-adenine adducts (or both) derived from BDO have been found in lung, liver, and testes of mice and in lung and liver of rats exposed to BD by inhalation (Koivisto et al 1996, 1997, 1998; Blair et al 2000). N7-(2,3,4-trihydroxybutyl)guanine adducts derived from BDO-diol have also been detected in liver DNA from mice and rats exposed to BD by inhalation (Tretyakova et al 1998; Blair et al 2000). Compared with rats, mice showed between twofold and threefold more liver DNA damage from these adducts.

Identifying the levels of BDO, BDO_2, and BDO-diol in humans would help to identify which rodent species may be the better model of cancer risks in humans. BD metabolism studies using human liver have suggested that humans are more like rats than mice in the amount and type of metabolites formed; however, considerable interindividual variation has been noted (Seaton et al 1995; Boogaard and Bond 1996; Boogaard et al 1996). Recent molecular dosimetry studies on DNA adducts of BD in rodents have shown results similar to those of analogous studies of Hb adducts of BD in rodents: BDO-diol was the major electrophilic metabolite of BD and accounted for approximately 98% of the DNA adducts formed (Koc et al 1999; Koivisto et al 1999; Boogaard et al 2000, 2001, unpublished results 2003; Swenberg et al 2000b; van Sittert et al 2000). This was a highly significant finding because (1) none of the earlier metabolism studies had measured BDO-diol, and (2) it is the weakest mutagen of the three electrophilic metabolites in that it is 1/200th as potent as BDO_2 (Cochrane and Skopek 1994a). Although BDO-diol can be formed by either oxidation of BD-diol or hydrolysis of BDO_2, the molecular dosimetry studies of DNA adducts strongly support hydrolysis as the primary pathway (Koc et al 1999; Boogaard et al unpublished results 2003). The Hb and DNA adduct data are consistent with the results of

metabolic studies in mice and rats, all of which indicate that mice are more efficient than rats in oxidizing BD to its electrophilic intermediates.

GENOTOXICITY OF BD

BD is unambiguously an animal carcinogen. This raises the question as to whether BD poses a cancer risk to humans. Although the studies of differential metabolism in animals help to identify the appropriate animal model to apply to human health risk assessment, it is even more fundamental to identify the underlying carcinogenic mechanisms. The degree to which animal carcinogenicity data may be extrapolated to humans depends in large part on these mechanisms, some of which may be unique to the test species. If a carcinogenic mechanism is unique to an animal test species, then the animal test results are not relevant for assessing risks to humans.

Most carcinogenic agents, however, produce cancer by mechanisms that are common to all species. One such mechanism is genotoxicity, in which the agent of concern is carcinogenic because it induces mutations in cancer-relevant genes or genetic regions in its target tissues.

The genotoxicity of BD metabolites has been demonstrated in all experimental systems that have provided metabolism (reviewed in de Meester 1988; Adler et al 1994, 1995a,b,c; Adler and Anderson 1994; Jacobson-Kram and Rosenthal 1995; Himmelstein et al 1997; Pacchierotti et al 1998; Jackson et al 2000). The mutagenicity of BD has been demonstrated many times over with the Ames test, in which metabolic activation is achieved by adding S9 fractions of liver from various species (deMeester et al 1980; Rosenthal 1985; deMeester 1988; Arce et al 1990; Norppa and Sorsa 1993; Jacobson-Kram and Rosenthal 1995). BDO_2 was the most potent mutagen when the three electrophilic BD intermediates were tested separately. BD metabolites have also induced gene mutations in eukaryotic microorganisms and insects (deMeester 1988), as well as in mammalian cells in vitro (Sernau et al 1986; McGregor et al 1991; Cochrane and Skopek 1993, 1994a,b; Recio et al 2000). Comparing the mutagenic potencies of BDO, BDO_2, and BDO-diol in cultured human cells showed that, on a molar basis, BDO_2 was 100-fold more potent than BDO and 350-fold more potent than BDO-diol (Cochrane and Skopek 1994a).

The kinds of mutational changes induced by BD metabolites in vitro have been studied by analyses of mutational spectra. Early studies showed increased frequencies of A→T base substitutions, which suggests that adenine adducts have a greater mutagenic potential than guanine adducts (Steen et al 1997a,b). More recent studies, however, found base substitutions of guanine in addition to the changes at adenine sites (Recio et al 2000). Of note, BDO_2, which preferentially induces A→T base substitutions in human cells, also significantly increased the frequencies of large gene deletions (Recio et al 2000). These spectra are consistent with the monofunctional or bifunctional alkylations expected by the BDO and BDO_2 metabolites, respectively.

Mammalian cells in culture, including those from humans, have also shown chromosome-level genetic changes following BDO or BDO_2 exposures (IARC 1999). BDO_2 was again the more potent of the two metabolites; effective BDO_2 concentrations for inducing SCEs were 10-fold lower in Chinese hamster ovary cells and 50-fold lower in human lymphocytes than BDO concentrations (Sasiadek et al 1991a,b). Detoxification of the BD metabolites is apparently important in protecting human cells from chromosome-level changes. Lymphocytes from individuals who were homozygous for the *GSTT1*-null allele (the allele that lacks function and cannot produce the GSTT1 isozyme) showed significantly higher percentages of SCEs following exposure to BDO_2 in vitro; this was not true for lymphocytes from individuals who are homozygous for the *GSTM1*-null allele (Kelsey et al 1995; Norppa et al 1995). By contrast, lymphocytes from individuals with the *GSTM1*-null genotype were more susceptible to SCE induction by BDO in vitro (Uuskula et al 1995), although neither *GSTT1* nor *GSTM1* genotypes affected SCE levels following exposure to BDO-diol in vitro (Bernadini et al 1996).

Mutation studies in animals exposed to BD in vivo allow comparisons with animal carcinogenicity data and potential extrapolations to humans (Recio et al 1992, 1998; Adler et al 1994; Cochrane and Skopek 1994b; Sisk et al 1994; Tates et al 1994; Recio and Meyer 1995; Meng et al 1998, 1999; Tates et al 1998; Walker and Meng 2000). Exposures of mice and rats to BD by inhalation have shown induction of somatic gene mutations (mostly at *HPRT*). In mice, the dose-response curves suggest that BD has greater mutagenic potency at low doses than at high doses (Walker and Meng 2000). In vivo studies using different BD metabolites have indicated that BDO_2 is the metabolite responsible for BD's in vivo mutagenicity at low exposure concentrations. Furthermore, molecular analyses of *HPRT* mutations showed a statistically significant increase in large deletions of genes in BD-exposed mice, which is also consistent with BDO_2 being the important mutagenic intermediate (Walker and Meng 2000).

Studies of rats exposed in vivo to BD showed significant increases in *HPRT* mutations at only the highest concentration (625 ppm; 1381.3 mg/m^3) of a multidose experiment;

the mutagenic potency in rats was 8.5-fold lower than in mice (Meng et al 1998, 1999; Walker and Meng 2000). Administration of BDO_2 also induced *HPRT* mutations in rats at even higher frequencies than it did in mice, which indicates that this metabolite is mutagenic in rats. However, direct administration of BDO_2 circumvented the metabolic pathways necessary to produce BDO_2 in vivo; and in rats exposed to BD (rather than directly to BDO_2), the formation of BDO_2 is minor (Thornton-Manning et al 1995).

Numerous investigators have measured BD-induced chromosomal changes in vivo in blood cells of mice (Irons et al 1987; Tice et al 1987; Jauhar et al 1988; Shelby 1990; Autio et al 1994; Xiao and Tates 1995; Xiao et al 1996; Stephanou et al 1997, 1998; reviewed in Pacchierotti et al 1998 and Jackson et al 2000). Significant increases have been observed for SCEs, micronuclei, and chromosomal aberrations. Dose-response relations were usually nonlinear (supralinear) (Autio et al 1994). BDO, BDO_2, and BDO-diol have also been shown to produce micronuclei in murine blood cells in vivo after injection (Xiao and Tates 1995; Adler et al 1997; Stephanou et al 1997, 1998; reviewed in Pacchierotti et al 1998). Again, BDO_2 was most effective, followed by BDO and BDO-diol. Significantly, chromosome-level genotoxic changes have never been demonstrated in vivo in somatic cells of rats following exposure to the parent compound BD (reviewed in Pacchierotti et al 1998). However, both the BDO and BDO_2 metabolites administered by injection did produce chromosomal changes in rats; once again, BDO_2 was more effective than BDO (Xiao and Tates 1995; Anderson et al 1997; Lähdetie and Grawé 1997). Again, direct administration of the metabolites circumvented the in vivo production in rats.

The germ cells of rodents exposed to BD or its metabolites or both also show genetic changes. Dominant lethality, an expression of chromosomal aberrations in germ cells, was shown in mice (Anderson et al 1996, 1998; Adler et al 1998) but not in rats (Anderson et al 1998). As was found for somatic cells, therefore, the germ cells of rats appear to resist chromosome-level genetic damage due to BD, although chromosome-level changes induced in vivo in germ cells by BD metabolites (as distinct from the parent compound) has been demonstrated in both species by many investigators (Adler et al 1995c, 1997; Xiao and Tates 1995; Anderson et al 1997; Lähdetie et al 1997).

In summary, the BD metabolites BDO, BDO_2, and BDO-diol are mutagenic in all systems; potencies follow the order of $BDO_2 > BDO > BDO\text{-diol}$. In vivo, both gene-level and chromosome-level mutations have been shown in mice, but only gene-level changes have been shown in rats following exposure to BD. Even at the gene level, BD's mutagenic potency is much greater in mice than in rats. In contrast to the parent compound BD, the metabolites of BD are mutagenic in vivo at both levels in both species. These observations are consistent with the results of metabolic studies, Hb adduct data, and the relative susceptibility to BD's carcinogenicity in the two species.

MOLECULAR EPIDEMIOLOGIC STUDIES IN HUMANS

Several health regulatory agencies, including the EPA, are considering methodologic modifications for assessing chemicals as carcinogens. As a complement to direct carcinogenicity data in animals or humans, a weight-of-evidence approach that includes noncancer endpoints is being considered. BD clearly causes cancer in animals and is genotoxic; therefore, it is a genotoxic carcinogen. With the newer methodologic approach, conclusive evidence of its genotoxicity in humans may be used to assess the risk of cancer in humans.

Molecular epidemiology is an emerging subdiscipline of epidemiology, which is the study of human populations. Rather than reporting disease distributions, molecular epidemiology seeks to detect deleterious environmental agents or their metabolites in accessible body matrices, measure the biological responses to these agents, and define genotypic or phenotypic factors that modify an individual's susceptibility to a specific agent. Molecular epidemiology might be thought of as the study of surrogates—surrogates of exposure, surrogates of effect, and surrogates of susceptibility—for the purpose of identifying causes of disease in humans, for predicting potential disease outcomes, for developing strategies for risk assessment, and, ultimately, for designing effective preventive measures.

Molecular epidemiologic studies of humans exposed to BD could provide evidence that this agent is metabolized to genotoxic intermediates, characterize this metabolism, and determine if overt genotoxicity can be demonstrated at the exposure levels encountered. Several such studies have now been completed and have provided preliminary data (see Table 1 and the references included in it) to compare with data from animals exposed to BD.

Several investigators have measured biomarkers of BD metabolism in subjects exposed in occupational settings. These biomarkers have included the urinary M1 metabolite and the Hb adducts HBVal and THBVal. One study of Texas monomer production workers showed that HBVal adducts are produced by an average BD exposure of 3.5 ppm (7.7 mg/m^3) at a rate of 0.004 pmol/g globin/hr (Osterman-Golkar et al 1993). In a study of Chinese workers,

Table 1. Summary Results from Studies of Four BD Worker Cohorts in Which Exposure Levels Were Compared with Biomarkers[a]

Endpoint	Texas Monomer Production Workers		Texas SBR Production Workers	Czech Republic Monomer and SBR Production Workers		Chinese Polymerization Workers
	Study 1	Study 2		Study 1	Study 2	
Urinary Metabolite						
M1	Dose-response elevation [Ward et al 1994]	Equivocal elevation [Ward et al 1996b]	Elevated with BD exposure [Ammenheuser et al 2001]			
Adducts						
Hb	Few workers showed elevation [Osterman-Golkar et al 1993]			Elevated HBVal with BD exposure [Sorsa et al 1994]		Elevated THBVal with BD exposure [Hayes et al 2000; Swenberg et al 2000a]
DNA					Elevated *N*-1-THB-A with BD exposure [Zhao et al 2000]	
Method of Assessing Somatic Gene Mutations						
Autoradiography	Dose-response elevation [Ward et al 1994]	Elevated with BD exposure [Ward et al 1996b]	Elevated with BD exposure [Ward et al 1996b, 2001; Ammenheuser et al 2001]			
Cloning			Elevated with BD exposure (mutation spectra consistent with BD effect) [Ma et al 2000]	No changes [Tates et al 1996]	No changes [Tates et al 1996]	No changes [Hayes et al 2000]
Chromosomal Mutations or Method of Assessing Them						
Aberrations				No changes; then elevated when reevaluated by *GST* status [Sorsa et al 1994, 1996]	Elevated with BD exposure [Šrám et al 1998]	
SCEs					Elevated with BD exposure [Šrám et al 1998]	No changes [Hayes et al 2000]
Micronucleus assay					No changes; also no changes by comet assay [Šrám et al 1998]	
Aneuploidy						No changes [Hayes et al 2000]
Challenge assay	Elevated with BD exposure [Au et al 1995]					

[a] Exposures were measured by personal monitoring, air monitoring, or both at a single time point, usually when biological samples were collected.

THBVal adduct concentrations were plotted against mean exposure levels for 33 workers (range 1–3.5 ppm) and 25 control subjects (0 ppm); the plot showed a regression slope significantly greater than 0 ($r^2 = 0.33$) (Swenberg et al 2000a). One point of note was that the THBVal adduct concentrations in this and other studies were occasionally higher than expected in unexposed control subjects; this suggests that either the adduct was not specific for BD, or it was endogenously produced, or both.

DNA adducts in BD-exposed workers have also been studied (Zhao et al 2000) (Table 1). Workers in a monomer production facility with BD exposures ranging from less than 0.02 to 37.6 mg/m^3 ($<$ 0.009 to 17.01 ppm) had *N*-1-(2,3,4-trihydroxybutyl)adenine (*N*-1-THB-A) adduct levels ranging from less than 0.1 (limit of detection [LOD]) to 25.0 adducts/10^9 nucleotides (mean of 4.5 adducts/10^9 nucleotides). By contrast, mean *N*-1-THB-A levels were significantly lower in the 11 unexposed control subjects. Adduct levels in exposed workers appeared to correlate with BD exposures.

Overt genotoxicity has also been investigated in BD-exposed humans with inconsistent findings. One laboratory conducted four studies of BD-exposed workers in Texas facilities (Osterman-Golkar et al 1993; Ward et al 1994, 1996b, 2001; Au et al 1995; Ma et al 2000; Ammenheuser et al 2001); three of these studies showed positive results using the autoradiographic assay for *HPRT* mutations and the fourth study (Ma et al 2000) showed positive results using the cloning assay for *HPRT* mutations. Mutational spectra data were compatible with the *HPRT* mutations being induced by exposure to BD in that an excess of deletions was found. By contrast, two studies in other laboratories, both using the cloning assay, failed to find increases in mutations in BD-exposed Chinese (Hayes et al 2000) and Czech (Tates et al 1996) workers even though the BD concentrations were similar to those in the studies of Texas workers.

Furthermore, no increases in chromosomal aberrations were found in the Texas facilities, although a challenge assay, possibly measuring unexpressed chromosomal damage, was positive (Au et al 1995). A study of Czech BD workers also failed to show evidence of BD-induced chromosomal aberrations, SCEs, or micronuclei on original analysis (Sorsa et al 1994). However, reanalysis with respect to *GST* genotypes showed that the BD-exposed workers who were homozygous for the *GSTT1*-null genotype had significantly higher aberration frequencies than the subjects who were not (Sorsa et al 1996). A later in vivo study of these Czech workers did show significant elevations in chromosomal aberrations and SCEs in the BD-exposed group (average 1.6 to 1.8 ppm [3.5 to 4.0 mg/m^3]) compared with the control workers (Šrám et al 1998). However, the study of Chinese BD polymerization workers referenced above produced negative results for SCEs and aneuploidy (Hayes et al 2000).

THE CURRENT STUDY

The current study was designed to address some of the significant knowledge gaps in the toxicity profile for BD in humans and to focus on methods that may be used in the future to evaluate human health risks. Definitive evidence for human carcinogenicity must come from human studies, which to date have produced only suggestive results. Interpretation of both the in vivo genotoxicity studies and the epidemiologic studies has been complicated by uncertainties about exposure doses and by potential confounders. Measures for overcoming these limitations were the focus of the current study.

One method for reducing uncertainties of exposure in epidemiologic studies is to use biomarkers to determine doses (Albertini 1998). The mere availability of biomarkers, however, does not mean that they will be useful for human studies directed at public health issues. Although the current armamentarium is large, few biomarkers have been validated to the point of known usefulness for epidemiologic studies (Albertini 1998). The human studies that assess and validate a biomarker have been termed "transitional" studies because they bridge the gap between purely laboratory investigations and field studies. A transitional study focuses on the biomarker, which is the dependent variable; the environmental exposure or adverse health outcome, which the biomarker is supposed to reflect or predict, is the independent variable. Such a study designed to validate a biomarker as a true measure of exposure or effect must first accurately measure the exposure or the health outcome because these are the standards against which the biomarker is assessed.

Several studies have measured biomarkers in humans occupationally exposed to BD (Table 1). However, in most instances, these assessments of biomarkers were useful to prove the principle that biomarkers could be identified rather than providing definitive validation of what the biomarker reflects. In terms of validating a biomarker as a surrogate measure of exposure, few if any of the studies thus far conducted have measured BD exposure levels exhaustively over time or at a given point in time determined to be appropriate for the biomarker being studied.

The current research was designed to rigorously evaluate several biomarkers for their utility as in vivo indicators of

BD exposure levels in workers and thereby validate them for use in future epidemiologic studies. To this end, exhaustive external measurements of the workers' exposures were made. We evaluated several conventional *biomarkers of exposure*: BD urinary M2 and M1 metabolite concentrations, and HBVal and THBVal Hb adducts. However, conventional *biomarkers of effect* also reflect internal doses of deleterious agents; thus, we evaluated several measures of genetic effects. These included *HPRT* gene mutations, measured by the two different assays used in past studies, and a variety of indicators of chromosomal damage. We also genotyped all subjects so we could evaluate certain genotypes as *biomarkers of susceptibility* that might indicate heightened individual responses to BD.

Although this study was designed to evaluate biomarkers only for their sensitivity in detecting BD exposures, the results provide additional evidence about the genotoxicity of BD for humans. Evidence for genotoxicity was evaluated in the context of (1) exhaustive external BD exposure measurements and (2) biomarkers of exposure that can corroborate the exposure measurements. This will add to the weight-of-evidence evaluations of BD's carcinogenicity or reproductive toxicity for humans.

SPECIFIC AIMS

The overall objective of this research was to rigorously evaluate a series of discrete nondisease biological endpoints (specifically, biomarkers in blood or urine) for their utility as in vivo indicators of BD exposure levels in the occupational setting. These endpoints represent points along a continuum from external BD exposure to the manifestation of disease. The overall research proceeded in two distinct phases. Phase I was a preliminary assessment to establish conditions for the definitive study in Phase II. Later, data and biological materials that had been collected in Phase II underwent supplemental analyses. Each phase and the supplemental analyses portion had its own set of specific aims.

PHASE I

Phase I began on 1 April 1997 and concluded on 31 March 1998. The final report for Phase I was submitted to the Health Effects Institute on 7 June 1998 and is not included here. Its specific aims were:

1. to identify potential factors that could compromise the definitive study;
2. to select a Study Coordinating Committee responsible for finalizing the definitive study protocol and analyzing the results;
3. to develop an exposure assessment protocol for Phase II; and
4. to propose a definitive protocol and budget for Phase II of the project.

PHASE II

Phase II began on 1 January 1998. Its specific aims were:

1. to precisely characterize BD exposures in workers by serial personal monitoring and replicate workplace area measurements in the industrial facilities near Prague;
2. to obtain blood and urine samples from each exposed worker and appropriate control subjects for immediate processing;
3. to measure biomarkers in laboratories with demonstrated expertise in the following specific assays:
   - biomarkers of exposure: concentrations of urinary M2 and M1 metabolites and of HBVal and THBVal Hb adducts;
   - biomarkers of effect (for validation as exposure surrogates): *HPRT* mutations in T cells determined by both autoradiography and cloning as evidence of

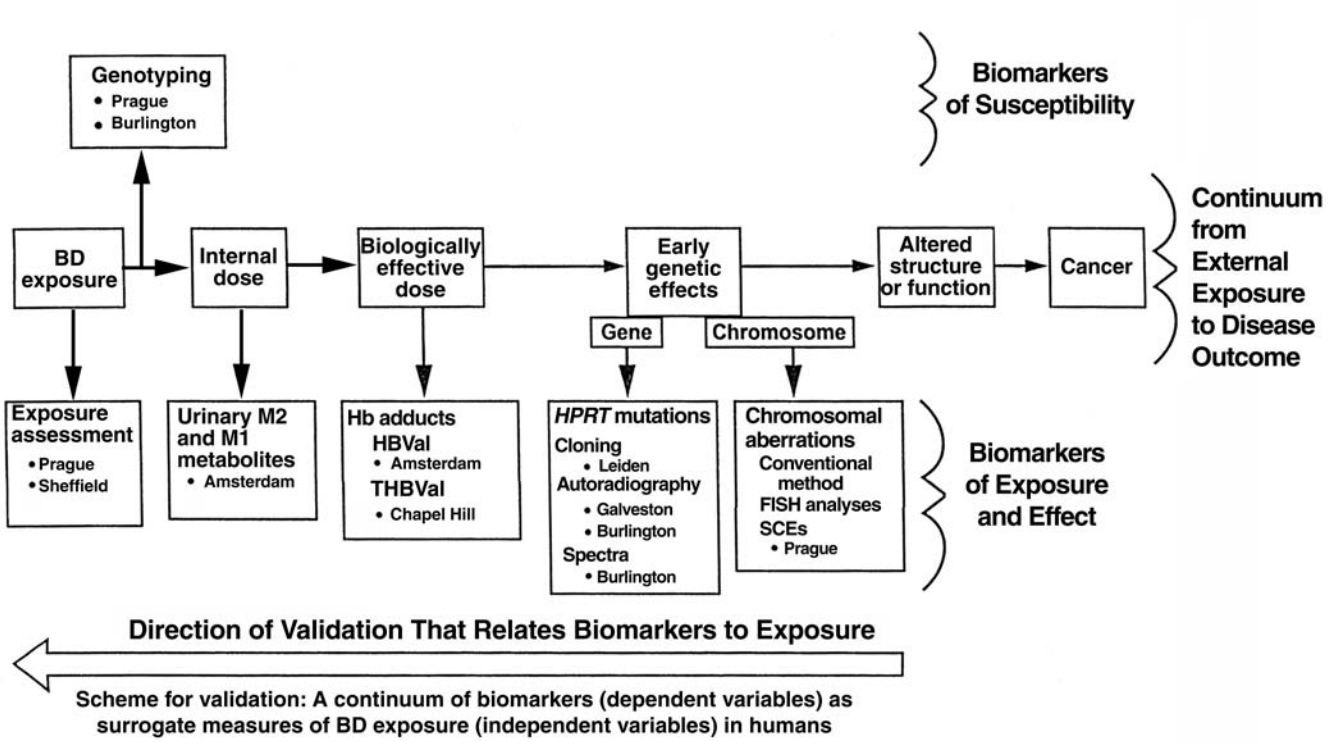

Figure 3. The biomarkers assessed in Czech workers exposed to BD according to the stage of exposure or effect being measured. Laboratories where each biomarker was analyzed are shown. Adapted from the Committee on Biological Markers of the National Research Council (1987).

somatic gene mutations; chromosomal aberrations (measured by the conventional method and by FISH) and SCEs as evidence of chromosome-level genetic changes;

- biomarkers of susceptibility: *glutathione*-S-*transferase M1* and *T1* (*GSTM1* and *GSTT1*) genotypes; and

4. to evaluate each biomarker in terms of its value for precisely reflecting external BD exposure levels in future epidemiologic studies by relating each biomarker to (a) measured exposures, (b) subjects' personal and medical characteristics obtained from questionnaires and employment records, and (c) the results from each other biomarker.

The biomarkers and the plan to validate them are depicted in Figure 3 according to the Biological Markers in Environmental Health Research (Committee on Biological Markers of the National Research Council 1987).

SUPPLEMENTAL ANALYSES

This portion of the overall investigation began 1 July 1999. In the supplemental studies we evaluated data and biological samples collected but not evaluated during Phase II. We also analyzed additional metabolic genotypes. The specific aims that were achieved in this portion were:

1. to statistically analyze selected questionnaire responses;
2. to analyze *HPRT* variant frequencies recalculated using the scoring performed in Burlington;
3. to genotype all study subjects for *CYP2E1*, *EH*, and *ADH* genetic polymorphisms;
4. to determine *HPRT* mutational spectra by molecular analyses of cryopreserved mutant lymphocyte isolates obtained from Phase II cloning assays; and
5. to relate workers' BD exposure levels calculated in Phase II to specific jobs and tasks so as to determine if such relations can be extended to other industrial sites where conventional epidemiologic studies have been conducted.

STUDY DESIGN: SUBJECT SELECTION, SAMPLE ACQUISITION AND HANDLING, AND STATISTICAL METHODS

This transitional epidemiologic study evaluated several biomarkers for their sensitivity to reflect known, low-dose BD exposure levels in occupational settings. The study focused on biomarkers, which were the dependent variables; BD exposures were carefully determined as the independent variables. The cross-sectional design was combined with a longitudinal component: BD exposures were measured at several time points before the biological samples were obtained to determine whether the biomarkers would reflect the exposures. Medical information was obtained for each subject to identify and adjust for factors that could modify the biomarkers' responses to BD exposure.

Eight institutions cooperated to accomplish the various stages and activities of this study (**bold type** indicates the place name used to identify each institution throughout the report):

- the BD and SBR manufacturing facilities near Prague, the Czech Republic; all exposed and control subjects were employed, exposures occurred in the course of work activities, exposure levels were assessed, questionnaires were completed, and biological samples were collected;
- the Laboratory of Genetic Ecotoxicology, **Prague**, the Czech Republic; biological samples were processed, coded, and cryopreserved; some biomarker assays were conducted; samples for other assays were shipped to other laboratories;
- Health and Safety Laboratory, **Sheffield**, United Kingdom; sorbent tubes used for personal monitoring were analyzed for concentrations of BD and other volatile organic compounds (VOCs);
- Leiden University, **Leiden**, The Netherlands; *HPRT* mutation assays were undertaken using the cloning protocol;
- University of Texas Medical Branch at Galveston, **Galveston**, Texas, USA; *HPRT* mutation assays were undertaken using the autoradiographic protocol;
- Shell International Chemicals, BV, **Amsterdam**, The Netherlands; urinary M2 and M1 metabolites and HBVal Hb adduct assays were conducted;
- University of North Carolina at Chapel Hill, **Chapel Hill**, North Carolina, USA; THBVal Hb adduct assays were conducted; and
- University of Vermont, **Burlington**, Vermont, USA; the central study office; procedures for genotyping subjects for *CYP2E1*, *ADH*, and *EH* were carried out; autoradiographic slides for *HPRT* variant frequencies were scored for verification; *HPRT* mutational spectra were determined; codes and data were maintained and analyzed; final reports were prepared.

The experimental protocol was approved by the Institutional Review Boards at both the Regional Institute of Hygiene of Central Bohemia and the University of Vermont, USA. All research was conducted in accordance with the Helsinki principles.

SUBJECT SELECTION AND EXPOSURE ASSESSMENT

All 83 subjects in this study were men employed by a single company. Two groups of workers with possible occupational exposures to BD were included: 24 in the BD monomer production facility (which comprised the entire production staff) and 34 in the BD polymerization facility (about half the work force). Of those not studied at the polymerization facility, most worked for an independent contractor who chose not to participate; a few were employed by the primary company and declined participation. The control group consisted of 25 administrative workers employed by the same company; they were selected because (1) they were male, and (2) they were willing to participate. No attempt was made to randomly select control subjects. (We had considered using power-plant workers as control subjects but were concerned that they may have some degree of exposure to other pollutants such as polycyclic aromatic hydrocarbons [PAHs].) All subjects received 1200 Czech crowns (~40 US dollars) as compensation for their participation. The research was explained to each participant in Czech and each one signed an Informed Consent form.

Workers' exposures were assessed during the period from 19 March through 12 June 1998. Personal monitors were used to assess BD exposure levels for all study subjects with multiple, semirandomly distributed measurements taken over a 60-day period for each exposed worker. The aim was to obtain BD exposure measurements near the beginning, middle, and end of each subject's assessment period. Exposure assessment also included concurrent levels of the VOCs benzene, toluene, and styrene. To supplement personal monitoring for BD and the VOCs, workplace area measurements were obtained from selected sites throughout the facilities. The details of the exposure assessment are provided in the later section Measurement of Occupational Exposure to BD.

Information about possible BD exposures before our assessment period was obtained from workplace measurements that had been made by the company during the

preceding five years and from personnel records of changes in workers' jobs. In addition, detailed employment histories for all subjects were obtained by questionnaires, administered by trained interviewers, which included questions about exposure to radiation, chemicals, and other hazardous materials in previous employment environments; recent exposures outside of work; smoking; alcohol and caffeine consumption; dietary habits; illnesses; and medications. Further health information for each subject was obtained from medical records in the company clinic, where all workers received their health care. Detailed information about the subjects is presented in the later section Workers' Exposure Groups, Personal Characteristics, and Metabolic Genotypes.

PROCEDURE TO ASSURE BLINDED ANALYSES

Each worker participating in the study was assigned a sequential identification number between 101 and 184 (the samples from subject 163 in the polymerization unit were lost to analysis). In addition, for each subject, a set of 11 unique random numbers between 1000 and 9999 was also generated and printed onto adhesive labels. One of these was affixed to each fraction of the blood and urine samples obtained from the subject before they were distributed to the laboratories doing the biomarker assays. Thus, each sample container had a label with a four-digit number so that the investigator performing the assay on that sample was blinded to which other samples were obtained from the same subject. A master list of the subject numbers and the 11 four-digit numbers assigned to the subject was kept in duplicate in Prague and Burlington. Codes were revealed to an assay investigator only after all assays by that investigator had been completed and the results had been transmitted to Burlington.

CHAIN OF CUSTODY

At the point of origin (Prague) and the time of each shipment, the four-digit random number identifying each sample or fraction being sent to each investigator in the shipment was listed on triplicate copies. Copy 1 was kept in Prague; copies 2 and 3 accompanied the shipment. When received, each investigator reviewed the samples and signed both copies; the investigator kept copy 2 and sent copy 3 to Burlington. The study office in Burlington made a fourth copy of the list; copy 3 was archived in the study's records and copy 4 was returned to Prague. In Prague, copy 4 was checked against copy 1, to ensure that all materials had been received and acknowledged by each investigator, thus completing the chain of custody for each sample.

Each investigator was responsible for the assay of each sample received. All samples were accounted for and all entries were complete at the time of data analyses for all samples. (If a sample had been lost, destroyed, or could not be analyzed, the study protocol specified a procedure through which an investigator would notify Burlington so that a proper adjustment could be made in the chain-of-custody records.)

SAMPLE ACQUISITION AND HANDLING

Subjects' Biological Samples

We obtained blood and urine samples for biomarker determination at the conclusion of each subject's exposure assessment period; blood was collected once on the last day and urine on six occasions: before and after workshifts on the last three days. Collections were scheduled for small groups of subjects on different days. Urinary metabolites are believed to be short-lived; this influenced our sample acquisition plan in two ways: First, we scheduled the first day of sampling after a two-day rest period to allow the retained metabolites from earlier exposures to fully wash out, which would ensure a purer before-shift urine sample. Second, we planned to measure BD exposure for each exposed worker on the day of sampling. However, timing the urine sampling to allow for the washout period and to coincide with exposure measurement proved difficult; thus, same-day exposure measurements were acquired for only a subset (5 monomer production workers + 15 polymerization workers = 20/58 = 34%) of the exposed workers and for 3 of the 25 (12%) control subjects.

The samples were transported on ice by automobile from the manufacturing facilities to the Prague laboratory within hours of collection, processed immediately, cryopreserved at −70°C, and maintained in the vapor phase of liquid nitrogen until and while they were sent to collaborating laboratories by air express in dry shippers.

Urine Urine samples from each worker were aliquoted into 50-mL fractions and frozen at −70°C. Each aliquoted fraction received a four-digit random identification number. Samples from day 1 were sent to Amsterdam where they were stored frozen at −20°C until analyzed; samples from days 2 and 3 were kept in Prague as a repository.

Blood Blood samples of approximately 60 mL per donor (later in the study, 100 mL) were collected in heparinized vacutainers (Greiner, Kremsmunster, Austria) in the medical unit of the industrial facility at the work site. At the Prague laboratory, a small amount (5–10 mL) of whole

blood from each subject was cultured immediately for cytogenetic analyses to be performed in Prague; these whole-blood samples were coded only with the subject's three-digit identification number. The remainder of each blood sample was fractionated into plasma, mononuclear cells (MNCs), and red blood cells (RBCs); each component was aliquoted for different biomarker assays. Each fraction or aliquot was assigned a random four-digit code.

Isolation of MNCs and Plasma Lymphocytes were separated from whole blood at room temperature using the LeucoSep system (Greiner), which consists of three components: a 50-mL tube, a porous filter disc, and histopaque. Histopaque (15 mL; density 1.077; Pharmacia, Peapack NJ) was pipetted into each LeucoSep tube and centrifuged for 30 seconds at 1000*g* to transfer the histopaque under the filter disc. The whole blood was transferred into one or more LeucoSep tubes and filled to the 50-mL mark with washing medium consisting of RPMI 1640 medium with 5% bovine calf serum (BCS). Blood and washing medium were mixed by inverting the tube a few times. Tubes were centrifuged for 10 minutes at 1000*g*. After centrifugation, erythrocytes were below the filter and the lymphocytes and other MNCs were just above the filter as a buffy coat. Each buffy coat was transferred to a 50-mL disposable tube, which was filled to the 50-mL mark with phosphate-buffered saline (PBS) (Sigma, St Louis MO). The tube was centrifuged for 10 minutes at 250*g*. The pellet was washed once more with PBS, mixed with medium A, which consisted of 80% RPMI 1640 and 20% BCS (Bioveta, Ivanovice ma Hane, the Czech Republic), and centrifuged for 10 minutes at 200*g*. The pellet was resuspended in 3 mL ice-cold medium A and mixed with 3 mL ice-cold medium B, which consisted of 80% BCS and 20% dimethylsulfoxide (DMSO). The final concentration of medium components in the lymphocyte suspension was DMSO 10%, RPMI 1640 40%, and BCS 50%. The ice-cold lymphocyte suspension was divided into 1.0-mL quantities, transferred to cryotubes, and kept on ice. Cryotubes were coded with random four-digit numbers, transferred from ice to a polystyrene box (with wall thickness of 2 cm), and stored frozen at −70°C until they were transferred in a dry shipper with liquid nitrogen to Leiden or Galveston.

When the whole blood was centrifuged in LeucoSep tubes, a large quantity of plasma collected on top of the buffy coat. The plasma from each donor was collected and stored at −70°C until it was shipped to Galveston in a dry shipper. Plasma samples were coded with the same random numbers as the MNC fractions from the same individual.

Isolation of Erythrocytes After the MNCs, plasma, and histopaque (Pharmacia) had been removed from the 50-mL LeucoSep tubes, the RBC layer remained. The RBCs were washed three times by adding 30 mL of isotonic saline to each 50-mL tube and centrifuging for 10 minutes at 485*g* at 4°C, after which the saline solution supernatant was aspirated and discarded. After the third wash, the RBCs were transferred to 2-mL vials, labeled with a four-digit random number, and stored at −70°C until shipped by dry shipper to collaborating laboratories. RBCs were sent to Amsterdam and Chapel Hill for determination of Hb adducts.

Sorbent Tubes for BD and VOCs

Diffusive thermal desorption tubes (PerkinElmer, Beaconsfield UK) prepared by Sheffield were used for personal monitoring and for the workplace area measurements. Each tube had its own engraved three-digit number and was marked with a red dot for BD and a green dot for VOCs. Workplace area monitors used pumps (type SKC) and sampling lasted for 15 minutes. After sampling, tubes were sent by Federal Express to Sheffield for analysis. Included with each shipment of 25 sample tubes was one blank tube and one positive control tube (using a short BD exposure), and the lists of numbers for the red and green tubes.

The original study design specified that BD exposure levels be monitored at 10 semirandomly spaced times for each exposed subject and once for each control subject, and that VOCs be monitored only once for several subjects; this plan would cover exposures for each individual over the course of 60 days. However, due to temporary shortages of available tubes resulting from turnover time lags between Prague and Sheffield, tubes were distributed as soon as they became available. Despite this change, each exposed subject was monitored for BD exposure at least once near the beginning of their own 60-day assessment period, several times at midterm, and for the last 3 days before blood was collected.

DATA PROCESSING AND STATISTICAL ANALYSIS

Data Processing

Subjects' questionnaires were mailed to Burlington, where they were screened for completeness and consistency; any problems were forwarded to Prague for resolution. Experienced data-entry personnel then entered the data into ASCII computer files directly from the questionnaires; this was done in duplicate and independently to verify data-entry accuracy.

Exposure and biomarker data from participating laboratories were transferred electronically to Burlington; most laboratories also mailed a diskette and printed copy of their data. Data were transferred from spreadsheets to ASCII data files and checked against printed copies of the

original files to verify the accuracy of data transfer. Each biomarker data file was then linked to a file containing the subject identification numbers corresponding to the random four-digit sample and aliquot numbers. For urinary metabolites, data from specimens obtained before and after the workshift were linked so that the results from each worker were contained on a single record.

Exposure results from Sheffield were matched to subject identification numbers and exposure assessment dates using the number etched on the sorbent tubes and the dates of shipment to Sheffield. For each tube, results provided in nanogram units were converted to TWA concentrations of BD or VOCs using the length of time in minutes that each exposure monitor was worn and the following equations supplied by Sheffield. The equations, as defined in Methods for the Determination of Hazardous Substances (MDHS) 63 (Bianchi et al 1996), are based on the amount in micrograms that is adsorbed in 400 minutes when the concentration is 2.5 ppm:

$$\text{ppm BD} = \text{ng} \times (400/\text{min}) \times (2.5/1.3) \times 0.001;$$

$$\text{ppm benzene} = \text{ng} \times (400/\text{min}) \times (2.5/1.72) \times 0.001;$$

$$\text{ppm toluene} = \text{ng} \times (400/\text{min}) \times (2.5/1.95) \times 0.001; \text{ and}$$

$$\text{ppm styrene} = \text{ng} \times (400/\text{min}) \times (2.5/2.15) \times 0.001.$$

An LOD of 1 ng was established for each measurement; this corresponded to TWA concentrations of 0.0016 ppm BD, 0.0012 ppm benzene, 0.0011 ppm toluene, and 0.0010 ppm styrene for an 8-hour workshift. Workers with exposures below 1 ng were given values of half the LOD, or 0.5 ng. Exposure concentrations in ppm were converted to mg/m^3 by multiplying each by the following conversion factor: BD, 2.21; benzene, 3.19; toluene, 3.77; and styrene, 4.26.

Exposure data were aggregated by subject to create a data file in which each record contained one subject's identification number and the dates and concentrations of his exposures in chronologic order. This file was linked to information on the dates of urine and blood sampling to calculate summary exposure metrics for each worker. Various exposure metrics were examined and the arithmetic mean was chosen for statistical analyses.

Workplace area BD and VOC concentrations were computed from the Sheffield results (provided in nanograms) and the rate in liters per minute at which air was pumped through the monitor during the 15-minute assessment period using the following equation:

$$\text{workplace area } mg/m^3 = \text{ng}/(\text{rate} \times 15 \text{ min}) \times 0.001.$$

Statistical Analysis

To characterize the variability in the many exposure measurements obtained for each worker, variance component analysis was performed using a random effects model with both day of exposure and subject as random effects (Lindsey 1999). Separate analyses were performed for the monomer and polymer groups. Frequency distributions and descriptive statistics of all summary exposure metrics and biomarkers were computed for each of the three exposure groups (administrative control subjects, monomer production workers, and polymerization workers). Variables with substantially skewed distributions were transformed (usually by taking the natural logarithm) to obtain approximate normality and all parametric statistical analyses were performed on the transformed values. For all analyses, P values of 0.05 and lower were considered to be statistically significant.

Differences among the three exposure groups for all exposure metrics and biomarkers were assessed both by one-way analysis of variance (ANOVA), using the Student-Newman-Keuls procedure for pairwise multiple comparisons, and by the nonparametric Kruskal-Wallis test, using Bonferroni-adjusted Mann-Whitney tests for pairwise comparisons (Montgomery 1991; Conover 1999). t Tests and Mann-Whitney tests were used to test differences between genotypes and between smokers and nonsmokers. Two-way ANOVA, with group as one factor and either genotype or smoking status as the other factor, was used to test for potential interactions between the effects of BD exposure and genotype or smoking on the biomarkers (Montgomery 1991). For most variables, the parametric and nonparametric tests gave very similar results. Because discrepancies occurred only when the assumptions of the parametric tests were violated, only the nonparametric results are presented in this report.

Bivariate relations between different biomarkers and between biomarkers and exposure variables were assessed by computing Spearman correlation coefficients to test whether larger values of one variable tended to be paired with larger values of the other variable, regardless of the functional form of the relation (Conover 1999). Pearson correlation coefficients were also computed to assess the linearity of relations. Unless otherwise noted, correlation results are presented as the Spearman coefficient (r). Regression analysis was used to quantify the relations between each Hb adduct and the average BD exposure. Multiple regression was used to examine the effects of exposure, as quantified by (a) average BD exposure, (b) average BD exposure 70 to 50 days before blood sampling, or (c) the Hb adducts, on the cytogenetic and *HPRT* mutation biomarkers after adjusting for age and smoking status.

Exposure group was included in some models to identify any group differences that were independent of BD exposure. For all regressions the exposure metrics and the Hb adducts were transformed by taking their natural logarithms. Transformations of the dependent variables were used when residuals were not normally distributed. For the cytogenetic marker of percentage of high-frequency cells, similar analyses were done using data from individual cells in a logistic regression with a random effect to represent the correlation between cells from the same individual (Bonassi et al 1999).

Multiple regression was also used to examine whether genotype modified the relation between BD exposure and biomarkers. As a first step, one-way ANOVA was used to test differences in biomarker levels among administrative control subjects with different genotypes, all of whom were assumed to have similar low-level exposures. If no differences were evident, a common intercept was assumed for all genotypes in regression analyses of data from exposed subjects. Genotype-exposure interaction terms were included to test for differences in slope. If genotype differences were evident in the control group, regression models for exposed workers also included genotype to allow for different intercepts.

Urinary metabolite data obtained at multiple time points (day 1 and day 3 before and after work) were analyzed by repeated-measures ANOVA using the logarithms of the four values as dependent variables. Day and time were the within-subject factors and group was the between-subjects factor. A similar analysis was performed on data from a subset of subjects for whom exposure had been measured on all three days of urine sampling to examine how the changes in urinary metabolites over time related to BD exposure. For all such analyses, BD exposure during the work period immediately preceding each urine sampling was included in the analysis as a time-varying covariate with the exception of the before-work sample on day 1. This sampling took place after a weekend away from work, so the prior exposure was assumed to be at an ambient environmental level of 0.011 mg/m^3 (0.005 ppm).

WORKERS' EXPOSURE GROUPS, PERSONAL CHARACTERISTICS, AND METABOLIC GENOTYPES

EXPOSURE GROUPS

Workplace Environment

The concentrations of BD exposure and the possibility of coexposure to other chemical agents are different in the several industries using BD. For example, BD monomers are produced in a closed process that allows less BD gas to escape into the workplace environment, whereas BD polymerization, as in SBR production, uses an open process that allows more BD and other gases to escape. Past BD exposure assessments have indicated that concentrations in the monomer production facility were routinely lower than those in the polymerization facility. Similarly, BD monomer production involves fewer coexposures to other agents, whereas polymerization uses numerous other chemicals. These differences may be one reason for the different epidemiologic findings between the two worker exposure groups (Delzell et al 1996b; Divine and Hartman 1996).

Total workplace exposure to BD is the sum of the background level in the plant plus task-related exposure (Lynch 2000). The background level of BD results from many small and large releases that occur throughout the plant. A worker moving about is continually exposed to this background level, which is higher or lower depending on the pattern of BD release and location in the plant. However, usually a worker's most intense exposure results from performing certain tasks; for example, when extracting samples, changing filters, preparing pumps for maintenance, and cleaning reactors, a worker is very close to the equipment and in intimate contact with any chemicals released. Such tasks are intermittent, often of short duration, and may be unscheduled and unpredictable. Details about workers' jobs and locations were provided by the company, including information about tasks performed within each job. However, specific tasks performed during the exposure assessment phase of this project were not recorded. Levels of exposure for different jobs were estimated by linking job titles, work locations, and task information with workers' exposure measurements.

Results: Job and Task Analysis

Table 2 shows the number of study subjects in the monomer production and polymerization exposure groups by job titles and work locations. The polymerization workers include those in the elastomer and latex units.

Table 2. Number of Exposed Subjects in Each Job and Work Location

Location[a]	Job Title: Operator	Pipe Fitter	Shift Foreman	Rotating Relief Worker
Monomer Unit				
All locations		3	5	
Control room	3			
Additive preparation	1			
Extraction	4			
Distillation	8			
Elastomer Unit				
All locations	1	5	1	1
Control room	2			
Polymerization line	4			
Latex degassing	1			
Degassing	1			
Absorption compressors	2			
Latex Unit				
All locations		1	2	1
Control room	3			
Additive preparation	4			
Polymerization line	3			
Degassing	1			
Absorption compressors	1			

[a] The elastomer and latex units comprise the polymerization unit.

Each worker's BD exposure was measured on approximately 10 days during a 60-day exposure assessment period and the mean of all measurements from workers with the same job title and work location were computed. The rank order of jobs by mean exposure from lowest to highest is shown in Table 3. In a study of BD exposure in US SBR plants (Macaluso et al 2000), past exposures were estimated by a complex method involving process history and mathematical modeling. That study ranked workers' BD exposure by task in the order shown in Table 4.

By comparing Tables 3 and 4, allowing for some differences in task nomenclature, we can understand worker exposures in terms of job, location, and task. Operators who spend their time in control rooms have background exposure but infrequent task exposures and are less exposed than operators who work in the various units close to the equipment. Workers in locations with large quantities of BD, such as the reactor hall, have higher exposures than those in locations where BD has been stripped from the latex. Closed-process units such as the monomer production unit have lower exposures than the more open-process polymerization units. Cleaning fouled equipment and sampling BD or latex rich in BD are sources of high exposure.

PERSONAL CHARACTERISTICS

Certain biomarkers examined in this research project, including the urinary metabolites and Hb adducts, are relatively specific for BD and are considered biomarkers of exposure. Two biomarkers of effect were also studied: the mutational endpoints, in which the frequencies of *HPRT* mutations in lymphocytes were examined, and the cytogenetic endpoints, in which several chromosomal changes were examined using a variety of techniques. Mutations and chromosomal changes are nonspecific and may be similarly affected by many genotoxic chemicals. *HPRT* mutational spectra were also determined in this study; they define kinds of mutational events that can be produced by classes of genotoxic agents rather than specific events caused by single chemicals. For these reasons, workers' employment histories, personal characteristics, coexposures to hazardous substances, and health histories were assessed by questionnaire to determine if any of these factors differed significantly among worker exposure groups or if any were correlated with given biomarkers.

Questionnaires

Questionnaires were developed in collaboration with project investigators and consultants. English versions were pretested by experienced interviewers at the Burlington Biometry Facility and a Czech version was then prepared by a translator in Prague. Subjects were interviewed and questionnaires completed by trained personnel from the Bohemian Department of Hygiene. All interviews were conducted in Czech; the time required was approximately 1 hour.

In the employment history portion of the interview, subjects were asked to describe the first job they had held for six months or longer, then all other jobs, and finally their current job. They provided the employer's name, a description of the work performed, the year they began and left each job, and the number of hours worked per week. If they held different jobs within the same company, they described each job. Subjects were then asked if they had been exposed to radiation or specific chemicals at any of

Table 3. Subjects' Jobs Ranked by BD Exposure

Job Title	Location	Task
Lower Exposure		
Operator	Monomer unit (control room)	Controls process
Pipe fitter	Monomer unit (all locations)	Installs and repairs pipes
Operator	Latex unit (degassing control room[a])	Controls process
Operator	Monomer unit (additive preparation)	Operates equipment, mixes additives
Operator	Elastomer unit (control room)	Controls process
Rotating shift foreman[b]	Latex unit (all locations)	Supervises unit operators and process
Shift foreman	Elastomer unit (polymerization)	Supervises unit operators
Rotating relief worker	Latex unit (all locations)	Operates and repairs equipment
Shift foreman	Monomer unit (all locations)	Supervises unit operators and process
Operator	Latex unit (degassing)	Operates equipment, monitors unit
Pipe fitter	Elastomer unit (all locations)	Installs and repairs pipes, welds
Operator	Monomer unit (extraction)	Operates extraction equipment
Day shift foreman[b]	Monomer unit (all locations)	Supervises process control
Pipe fitter	Monomer unit (all locations)	Installs and repairs pipes, operates and cleans equipment
Medium Exposure		
Operator	Latex unit (polymerization control room[a])	Controls process, collects BD samples
Pipe fitter	Elastomer unit (all locations)	Installs and repairs pipes
Shift foreman	Latex unit (polymerization unit[c])	Supervises unit operators
Operator	Elastomer unit (latex degassing)	Operates equipment, monitors unit
Operator	Latex unit (absorption compressors)	Operates equipment, collects samples
Operator	Elastomer unit (polymerization line)	Operates equipment, collects samples
Operator	Elastomer unit (control room)	Controls production
Cleaner[b]	Elastomer unit (all locations)	Flushes coagulate from vessels and pipes
Operator	Latex unit (polymerization line)	Operates equipment, collects samples, mixes additives
Operator	Monomer unit (distillation)	Operates distillation equipment
Operator	Elastomer unit (degassing)	Operates equipment, monitors unit
Operator	Elastomer unit (polymerization hall[c])	Operates equipment, collects samples, feeds additives
Higher Exposure		
Rotating relief worker	Elastomer unit (all locations)	Operates equipment, collects samples
Operator	Elastomer unit (absorption compressors)	Operates equipment, collects samples
Pipe fitter	Latex unit (polymerization unit[c])	Installs and repairs pipes, adjusts and operates equipment

[a] Included under "control room" as a location in Table 2.

[b] Included under "shift foreman" as a job title in Table 2.

[c] Included under "polymerization line" as a location in Table 2.

their previous places of employment. They were also asked about specific exposures outside of work (such as those associated with hobbies, gardening, and home or automobile repair) during the previous 3 months.

The smoking history portion of the questionnaire included questions about passive exposure to tobacco smoke, both at work and outside of work. Subjects were also asked about the amount of alcohol and caffeine they consume; the number of servings of fruit, vegetables, and meats they have each week; and their use of specific medications. A separate questionnaire was designed to elicit medical information about chronic illnesses, recent acute illnesses, diagnostic x-rays, and medical therapies. This information was provided by physicians responsible for the workers' health care, who completed a questionnaire for each subject from medical records in the company clinic.

Table 4. United States Rubber Reserve Workers' Tasks Ranked by BD Exposure

Level of Exposure	Task
Lower	Sample styrene shipments
	Clean stripping column
	Perform minor maintenance of styrene pumps
	Draw off water from recycled-styrene tanks
	Open heat exchangers
	Perform minor maintenance of unit
Medium	Change small filters
	Monitor transfers from pump house
	Inspect unit for leaks or minor problems
	Open blow-down manholes
	Drain large filters
	Open flash-tank manholes
	Perform minor maintenance of BD pumps
	Perform minor maintenance of recovery compressors
Higher	Clean reactor vessel
	Clean blow-down tank
	Open reactor manholes
	Clean flash tank
	Test BD inhibitor (process control)
	Sample BD

Table 5. Demographic Information on Workers

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)
	Mean ± SD	Mean ± SD	Mean ± SD
Age	39.5 ± 13.4	40.6 ± 13.1	39.3 ± 12.1
Years at company	12.4 ± 12.3	18.3 ± 13.7	15.1 ± 11.2
Years in district	27.7 ± 13.3	33.0 ± 11.9	30.6 ± 11.9
	n (%)	n (%)	n (%)
Education[a]			
<12 Years	1 (4)	16 (67)	24 (70)
12 Years	12 (48)	8 (33)	8 (24)
>12 Years	12 (48)	0 (0)	2 (6)

[a] Groups differ significantly at $P < 0.05$ by χ^2 test.

Results: Characteristics and Coexposures

Analysis of questionnaire data revealed the three exposure groups to be fairly well balanced with respect to age, years of employment by the company, and years of residence in the district (Table 5). Mean ages were 39.5, 40.6, and 39.3 for control, monomer, and polymer groups, respectively. Mean years employed by the company were 12.4, 18.3, and 15.1; and mean years of residence were 27.7, 33.0, and 30.6 for the three exposure groups, respectively. Educational status, however, was significantly different among the three exposure groups: the control group had a higher mean number of years in school (48% with more than 12 years) than either the monomer group (0% with more than 12 years) or the polymer group (6% with more than 12 years) ($P < 0.05$ by χ^2 test).

Subjects' health habits, medication usage, and health histories are summarized in Table 6. In the control group, one current nonsmoker did not provide information about his smoking history and one subject did not provide information about his current smoking status. Therefore, $n = 23$ for this characteristic. For later analyses in which *current smoking* is a factor, n values are 9 smokers, 15 nonsmokers, and 24 instead of 25 for the total control group. Fewer control subjects were current smokers (39% versus 54% and 47% for the monomer and polymer groups, respectively) but the differences were not statistically significant; in all exposure groups at least 70% of the workers had smoked at some time during their lives. As a consequence, we found no significant difference among the exposure groups in total cigarette consumption as measured by pack-years (the number of packs of cigarettes smoked per day multiplied by the number of years smoked). Average pack-years for the control, monomer, and polymer groups were 9.9, 8.4, and 13.1, respectively. All workers (100%) in the monomer group spent time in the presence of other smokers at work or outside of work, compared with 72% of the control group and 74% of the polymer group ($P < 0.05$ by χ^2 test).

The three exposure groups were similar in their dietary habits, including alcohol and caffeine consumption, except that the average number of vegetable servings per week was significantly higher in the control group ($P < 0.05$ by ANOVA). Subjects in the control group were also more likely to take vitamins regularly and to have had a vaccination within the past year ($P < 0.05$ by χ^2 test).

The medications most frequently used during the previous 3 months were antibiotics, cough or cold remedies, and blood pressure pills; usage was similar among the three exposure groups (Table 6). A number of subjects also reported taking medications other than those specified; the proportion of these subjects was highest in the monomer group ($P < 0.05$ by χ^2 test). A higher proportion of control subjects had an infectious illness during the previous 3 months ($P < 0.05$ by χ^2 test), but the prevalence of chronic

Table 6. Workers' Health Habits, Medications, and Health Histories

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)
	n (%)	n (%)	n (%)
Health-Related Factors			
Smoking status[a]			
Current smoker	9 (39)[a]	13 (54)	16 (47)
Exsmoker	7 (30)[a]	5 (21)	9 (26)
Never smoked	7 (30)[a]	6 (25)	9 (26)
Passive smoke exposure[b]	18 (72)	24 (100)	25 (74)
Take vitamins regularly[b]	10 (40)	3 (12)	6 (18)
Vaccination in past year[b]	8 (32)	1 (4)	4 (12)
	Mean ± SD	Mean ± SD	Mean ± SD
Food and Beverage Consumption per Week			
Fresh fruit servings	6.1 ± 8.1	4.3 ± 2.8	4.7 ± 2.4
Vegetable servings[c]	5.4 ± 1.9	3.6 ± 2.6	4.2 ± 2.3
Red meat servings	2.7 ± 2.5	2.9 ± 2.3	2.6 ± 2.2
Caffeinated beverages	19.4 ± 14.2	25.9 ± 16.3	19.6 ± 13.9
Alcoholic beverages	7.7 ± 8.3	8.1 ± 7.1	7.8 ± 6.5
	n (%)	n (%)	n (%)
Medications Taken During the Previous 3 Months			
Antibiotics	1 (4)	3 (12)	4 (12)
Cough/cold remedies	6 (24)	6 (25)	10 (29)
Antihistamines/ allergy remedies	0 (0)	0 (0)	0 (0)
Analgesics/ antiphlogistics	3 (12)	0 (0)	1 (3)
Antidepressants	0 (0)	0 (0)	0 (0)
Blood pressure pills	5 (20)	5 (21)	2 (6)
Hormones	0 (0)	0 (0)	0 (0)
Steroids	0 (0)	0 (0)	0 (0)
Immunosuppressants	0 (0)	0 (0)	0 (0)
Antiolytics/ neuroleptics	0 (0)	0 (0)	0 (0)
Antiepileptics	0 (0)	0 (0)	0 (0)
Diet pills	0 (0)	0 (0)	0 (0)
Sleeping pills	1 (4)	0 (0)	0 (0)
Other[b]	4 (16)	9 (37)	3 (9)

(Table continues next column)

[a] In the control group, one subject did not provide information on his current smoking status and one current nonsmoker did not provide information about his smoking history. Therefore, n = 23 for this characteristic. For later analyses in which *current smoking* is a factor, n values are 9 smokers, 15 nonsmokers, and 24 instead of 25 for the total control group.

[b] Groups differ significantly at $P < 0.05$ by χ^2 test.

[c] Groups differ significantly at $P < 0.05$ by ANOVA.

[d] Information on high blood pressure was missing for one monomer production worker; therefore, n = 23 for this characteristic.

Table 6 (*Continued*). Workers' Health Habits, Medications, and Health Histories

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)
	n (%)	n (%)	n (%)
Health History Information			
Heart disease	3 (12)	1 (4)	2 (6)
Diabetes	0 (0)	0 (0)	0 (0)
Hepatitis	1 (4)	1 (4)	4 (12)
High blood pressure	5 (20)	5 (22)[d]	3 (9)
Thyroid disease	0 (0)	1 (4)	1 (3)
Anemia	2 (8)	0 (0)	4 (12)
Asthma	1 (4)	0 (0)	0 (0)
Lupus	0 (0)	0 (0)	0 (0)
Heart disease	3 (12)	1 (4)	2 (6)
Rheumatoid arthritis	0 (0)	1 (4)	2 (6)
Other arthritis	1 (4)	2 (8)	4 (12)
Liver disease	2 (8)	2 (8)	2 (6)
Kidney disease	2 (8)	2 (8)	4 (12)
Mononucleosis	2 (8)	1 (4)	1 (3)
Cancer	1 (4)	1 (4)	0 (0)
Infectious illness in past 3 months[b]	18 (72)	9 (37)	17 (50)
Diagnostic x-ray in past year	18 (72)	11 (46)	20 (59)

illness was similar among the three exposure groups (Table 6). One control subject and one monomer production worker had been diagnosed with cancer; neither had been treated with radiation or chemotherapy. Diagnostic x-rays had been performed within the previous year on 72% of administrative control subjects, 46% of monomer production workers, and 59% of polymerization workers; these differences were not statistically significant.

When questioned about other exposures to chemicals (Table 7), 60% of administrative control subjects, 79% of monomer production workers, and 53% of polymerization workers reported occupational exposure to hazardous substances before their current employment. The most common type of previous exposure was to solvents or degreasers. We noted only one significant difference among the groups in the types of previous exposure: 25% of administrative control subjects and 21% of polymerization workers, but no monomer production workers, had been exposed to nonspecific dust ($P < 0.05$ by χ^2 test).

Questions about exposure to chemical products outside of work during the previous 3 months revealed that a significantly higher proportion of monomer production workers (67%) used such products compared with 28% of administrative control subjects and 35% of polymerization

Table 7. Other Exposures to Chemicals

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)
	n (%)	n (%)	n (%)
Occupational Exposures Before Current Employment			
Radiation	2 (8)	0 (0)	0 (0)
Asbestos	2 (8)	1 (4)	4 (12)
Coal products	1 (4)	2 (8)	1 (3)
Pesticides/herbicides	1 (4)	0 (0)	1 (3)
Metal fumes/particles	3 (12)	3 (12)	6 (18)
Gases	1 (4)	0 (0)	3 (9)
Petroleum products	3 (12)	3 (12)	3 (9)
Dyes	2 (8)	3 (12)	4 (12)
Solvents/degreasers	4 (17)[a]	4 (17)	9 (26)
Paints/stains/varnish	3 (12)	3 (12)	4 (12)
Dusts[b]	6 (25)[a]	0 (0)	7 (21)
Other chemicals	3 (12)	0 (0)	3 (9)
Exposure Outside Work During the Previous 3 Months			
Pesticides/herbicides	4 (16)	2 (8)	1 (3)
Paint/turpentine/ varnish[b]	10 (40)	2 (8)	13 (38)
Glues/adhesives	2 (8)	0 (0)	0 (0)
Lead	3 (12)	1 (4)	2 (6)
Oils/gasoline[b]	11 (44)	4 (17)	17 (50)
Hair dyes	0 (0)	0 (0)	0 (0)
Solvents	4 (16)	3 (12)	4 (12)
Fumes/gases	4 (16)	0 (0)	2 (6)
Other chemicals	0 (0)	1 (4)	0 (0)

[a] Information on exposure to solvents/degreasers and exposure to dusts was missing for one control subject; therefore n = 24 for these characteristics.

[b] Groups differ significantly at $P < 0.05$ by χ^2 test.

workers ($P < 0.05$ by χ^2 test). Again, the types of exposures were fairly similar (Table 7), but fewer monomer production workers had exposure to paints or varnishes and to oils or gasoline ($P < 0.05$ by χ^2 test).

Of 110 questionnaire variables pertaining to health and possible toxic exposures outside of work, responses for 11 variables were significantly different among the three exposure groups. These included (1) the health-related and dietary variables of taking medications or vitamins, having vaccinations, consuming tea and vegetables, and having a recent infectious illness; and (2) prior possibly toxic exposures to dusts, paints, oil, and passive smoke, or unusual exposures at work. These 11 statistically significant differences are more than the five or six that would be expected due to chance if the three groups of workers came from the same population and the variables being tested were truly independent. Many of the variables are, in fact, correlated and some, such as passive exposure to cigarette smoke, are related to the different work settings of the three exposure groups. Thus, it is difficult to determine the number of differences that would be expected to arise from random variation.

We found a significant difference among exposure groups on the basis of which of two interviewers administered the questionnaires ($P < 0.05$ by χ^2 test). One interviewer worked with all subjects in the monomer production facility (100%) and most subjects in the polymerization facility (65%); the other worked with all but one of the administrative control subjects (96%). This assignment of interviewers could have introduced bias in the information about job histories and exposure to toxic chemicals outside of the workplace. Most of the health information, however, was obtained directly from medical records by a physician in the company clinic and therefore is independent of the assigned interviewers.

METABOLIC GENOTYPES

How specific and nonspecific biomarkers respond to BD exposure can be influenced by metabolic genotypes. [*Note that all discussions of genotyping and other genetic content use nomenclature that was accurate at the time of this study; as the field of genetics continues to evolve, the names and assigned locations of some genes are changing.*] As described in the Overview, BD itself is not genotoxic, but must be metabolized in vivo to its genotoxic metabolites, which produce DNA damage. The activities of several enzymes mediate this metabolism. The oxidation reactions depend on at least two CYP-dependent monooxygenases: CYP2E1 and CYP2A6 (Okino et al 1987). In vivo detoxification of BD metabolites depends on three enzymes: EH, ADH, and GST.

CYP2E1 is a major enzyme expressed in liver and other tissues; it can be induced by ethanol and other chemicals, and its activity varies widely among individuals (Guengerich and Turvy 1991; Lucas et al 1995). CYP2E1 is known to be involved in the metabolism of many chemicals, such as PAHs and nitrosamines, and can convert BD to BDO, and BDO to BDO_2 in humans (Seaton et al 1995). Seaton and colleagues (1995) found a 60-fold variation in enzyme activity in human tissue samples that varied with CYP2E1 protein levels, which suggests a genetic component to the enzyme's activity.

The *CYP2E1* gene, which is located at chromosome 10q24.3 (on the long arm of chromosome 10 at band 24.3), is known to have several polymorphisms. The *Rsa*I and *Pst*I polymorphisms in the 5′ flanking region of the gene

(alleles *C1* and *C2*) are in complete linkage disequilibrium (Watanabe et al 1990) and are known to affect gene transcription (Hayashi et al 1991). The *C2* allele codes for increased enzymatic activity. In addition, the A•T polymorphism in intron 6 causes the loss of a *Dra*I restriction site (alleles *C* and *D*) (Uematsu et al 1991). Associations of these polymorphisms with susceptibility to certain cancers has been reported by some researchers (Yu et al 1995; Shields et al 1996; El-Zein et al 1997; Le Marchand et al 1998; Lin et al 1998), although others have not found these relations (Hirvonen et al 1993). For example, Lin and coworkers (1998) found that *C1*/*C1* individuals had a fivefold increased risk of esophageal cancer; and Tan and associates (2000) found an increased frequency of the *C1* allele in patients with esophageal cancer. However, another study found that risk of airway cancer doubled in patients with the *CYP2E1 C* allele and the *C2* allele (Bouchardy et al 2000). A decrease in gastric cancer has been associated with the *C2* allele (Nishimoto et al 2000), but no effect of *CYP2E1* genotype was found on hepatocellular carcinoma (Wong et al 2000b). The *C2* allele produced an odds ratio of 1.91 with colon cancer (Kiss et al 2000). Kim and associates (1994), however, found no difference in *CYP2E1* activity in response to chlorzoxazone among the different genotypes in 55 white subjects, although only one individual with the *C1*/*C2* genotype and one individual with the *C*/*C* genotype were identified. The importance of these polymorphisms to the formation of BDO and BDO_2 is thus uncertain; although no difference in response to chlorzoxazone was reported, differences in response to BD may exist among the different *CYP2E1* genotypes.

EH is located at 1q24.1 (the long arm of chromosome 1); it has two polymorphic sites (amino acids 113 and 139) that have variants with sufficiently high frequencies to be detected in small-group studies (Hassett et al 1994). Substituting histidine for tyrosine at position 113 in exon 3 (*His113*) results in a 40% decrease in EH enzyme activity compared with the tyrosine allele (*Tyr113*). Substituting arginine for histidine at position 139 in exon 4 (*Arg139*) produces approximately a 25% increase in enzyme activity compared with the histidine allele (*His139*) (Hassett et al 1994). A number of studies have found associations between these alleles and cancer: *His113* with an increase in aflatoxin-associated hepatocarcinoma, but also with a decrease in ovarian cancer (McGlynn et al 1995; Lancaster et al 1996); increased relative risk of lung cancer with increased EH activity (Benhamou et al 1998); a high number of individuals carrying the *Arg139* mutation in a group with hepatocellular carcinoma (Wong et al 2000a); increased EH activity with squamous cell carcinoma (Lin et al 2000), whereas decreased EH activity with colon cancer (Harrison et al 1999); and *EH* genotypes with slow hydroxylation and high *HPRT* mutant frequencies (Viezzer et al 1999).

ADH is a family of isoforms (different forms of the ADH protein) that catalyze the oxidation of alcohols and ketones. *ADH1* is not polymorphic in humans but *ADH2* and *ADH3*, both located at chromosome region 4q22, are polymorphic (Xu et al 1988; Matsuo et al 1989; Osier et al 1999). *ADH2* has a well-known polymorphism (*ADH2*2*; *His47* instead of *Arg47*) that is found at much lower frequencies in people of Chinese origin who have alcoholism (Muramatsu et al 1995; Higuchi et al 1996). The maximal velocity of enzyme reaction (V_{max}) for the *ADH2*2* allele is 40-fold greater than for the *ADH2*1* allele (Edenberg and Bosron 1997). Another rare allele, *ADH2 Indianapolis* (*Cys369* instead of *Arg369*), is found primarily in black populations (Bosron et al 1980; Xu et al 1988). One study found no relation between *ADH2* genotype and hepatocellular carcinoma (Takeshita et al 2000), although a relation between esophageal cancer and *ADH2* genotype has been reported (Hori et al 1997; Yokoyama et al 2001). Two polymorphisms at the *ADH3* locus are in tight linkage disequilibrium resulting in two common alleles: The *ADH3*1* allele has *Arg271* and *Ile349*, whereas the *ADH3*2* allele has *Gln271* and *Val349*. The enzyme produced by *ADH3*1* has a higher V_{max} (about 2.5-fold) than *ADH3*2* (Edenberg and Bosron 1997). As with the *ADH2*2* allele, higher frequencies of the *ADH3*2* allele have been found in people of Chinese origin who have alcoholism (Higuchi et al 1996; Osier et al 1999). Increased risk of breast cancer has been reported for premenopausal women with the *ADH3*1/*1* genotype (Freudenheim et al 1999), although the *ADH3* genotype has not been associated with cancer in the upper airways (Bouchardy et al 2000).

GST is also a family of isoforms that catalyzes the addition of GSH to a number of carcinogens to form thioesters, a first step in detoxification. There are five *GSTM* genes that map to 1p13.3, of which *GSTM1* and *GSTT1* are the most relevant to this study. The *GSTM1* gene has a high-frequency (0.4–0.7) null allele (an allele that does not allow the GST enzyme to be produced) in most populations around the world (Board et al 1990), which is the result of crossing over between the homologous *GSTM* genes (Xu et al 1998). The *GSTM1* gene also has two common coding polymorphisms, *GSTM1*A* and *GSTM1*B*, which differ by a single base in exon 7 (Fryer et al 1993). The *GSTM1*-null allele has been associated with increased cancer risk (including lung [Seidegard et al 1990; Charrier et al 1999], colon [Zhong et al 1993], cervical [Kim et al 2000; Malats et al 2000], lung and bladder [Salagovic et al 1998], and oral cavity [Katoh et al 1999] cancers) and with

higher DNA adduct levels in people who live in polluted areas of Poland (Butkiewicz et al 1999). Individuals who have *GSTM1*A/*B* had a reduced risk of upper aerodigestive tract cancer (Matthias et al 1998).

The *GSTT1* gene (chromosome location 22q11.2) also has a null allele that occurs with relatively high frequency in certain people (approximately 40% in white populations) (Pemble et al 1994). *GSTT1*-null genotypes are associated with cervical, renal, and bladder cancer, and with adult leukemia (Salagovic et al 1998; Kim et al 2000; Rollinson et al 2000; Sweeney et al 2000), but not with breast cancer (Curran et al 2000) or lung cancer (To-Figueras et al 1997). Subjects with the *GSTM1*-null or *GSTT1*-null genotype had reduced risk of relapse of childhood acute lymphoblastic leukemia (Stanulla et al 2000). When infants with leukemia were studied (Garte et al 2000), the deletion of *GSTM1* and *GSTT1* from their parents' genotypes was significantly higher than expected. Subjects who were null for both *GSTM1* and *GSTT1* had an odds ratio of 3.64 for head and neck cancer (Cheng et al 1999). Having a *GSTT1*-positive genotype and the *GSTM1*-null phenotype increased the number of DNA adducts in coke oven workers (Viezzer et al 1999).

Subjects in the current study were genotyped for *CYP2E1*, *EH*, two isoforms of *ADH* (*ADH2* and *ADH3*), *GSTM1*, and *GSTT1*, to determine if these might be genetic susceptibility factors that influence biomarkers of BD exposure.

Genotyping Methods

GSTM1* and *GSTT1 Genotyping for *GSTM1* and *GSTT1* was performed in Prague using MNCs isolated from fresh blood samples. The *GST* deletions within these genes were identified using a polymerase chain reaction (PCR) protocol for simultaneous analysis with primers (Zhong et al 1993; Premble et al 1994). This PCR assay employs five oligonucleotide primers (*T1*, *T2*, *M1*, *M2*, and *M3*); *T1* and *T2* are homologous to sequences in the *GSTT1* gene, *M1* and *M2* are homologous to sequences in both the *GSTM1* and *GSTM4* genes, and *M3* is unique to sequences of the *GSTM1* gene (Genset, Paris France). Sequences of these primers are:

T1	5′-TTCCT TACTG GTCCT CACAT CTC-3′
T2	5′-TCACC GGATC ATGGC CAGCA-3′
M1	5′-CGCCA TCTTG TGCTA CATTG CCCG-3′
M2	5′-ATCTT CTCCT CTTCT GTCTC-3′
M3	5′-TTCTG GATTG TAGCA GATCA-3′

Using the five primers in a single amplification reaction, the *T1*/*T2* primer pair yields the *GSTT1* fragment of 480 bp, the *M1*/*M2* primer pair anneals to sequences in both the *GSTM1* and *GSTM4* genes to yield fragments of 157 bp, and the *M1*/*M3* primer pair generates a product of 230 bp from the *GSTM1* gene only.

DNA for analysis was extracted from MNCs from study subjects by standard methods. The PCR master mixture (total volume of 50 μL) contained 2 mM magnesium chloride ($MgCl_2$; Promega, Madison WI), 0.16 mM deoxynucleoside triphosphates (dNTPs; Fermentus, Vilnius Lithuania), 10 pmol primer *T1*, 18 pmol primer *T2*, 32 pmol primer *M1*, 16 pmol primer *M2*, 16 pmol primer *M3*, and 1.5 U *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) in Promega buffer (50 mM potassium chloride [KCl], 10 mM Tris-hydrochloric acid [HCl] [pH = 8], and 1% Triton X-100) 1×; to this mixture was added 100 ng DNA in 50 μL H_2O. PCR conditions included an initial 4 minutes at 94°C for primary denaturation, then 36 cycles of: 57°C for 1 minute for annealing, 72°C for 1 minute for extension, and 94°C for 1 minute for denaturing; and a final cycle for 5 minutes at 72°C for the final elongation. Amplification products were made visual by mixing 15 μL of PCR product with loading buffer and performing electrophoresis on the samples on 2% agarose gel with ethidium bromide (25 μg/100 mL of gel). Electrophoresis, using Maxi Agagel (Biometra, Göttingen Germany) was carried out at 90V for 1.5 hours. The products were made visual with UV illumination and a photograph was recorded.

Assays were repeated twice for each sample. If the results differed, the assay was repeated again. Results of all replicates are reported. Amplification of a region of the *GSTM4* gene served as the internal control for the PCR reaction. A DNA size marker, pUC mix (MBI Fermentas, Vilnius, Lithuania; 0.5 μg/mL, 1 μL mixed with 4 μL of loading buffer), was loaded together with samples to accurately identify the sizes of the PCR products. Only fragments of the same intensity of luminescence were evaluated.

Amplification of DNA from individuals possessing two wild-type *GSTM1* and *GSTT1* alleles yielded PCR products of 480, 230, and 157 bp. DNA from individuals who are homozygous for both the *GSTM1* and *GSTT1* gene deletions generated only the 157-bp product from the *GSTM4* gene. DNA from individuals lacking the *GSTM1* alleles but possessing the *GSTT1* gene generated fragments of 480 and 157 bp. Amplification of DNA possessing only the *GSTM1* gene yielded fragments of 230 and 157 bp. Individuals who are heterozygous for *GSTM1* or *GSTT1* or both alleles could not be distinguished from individuals with homozygous wild-type genotypes using this assay.

ADH2, ADH3, EH, and CYP2E1 Genotyping for the *ADH2*, *ADH3*, *EH*, and *CYP2E1* polymorphisms was performed in Vermont using DNA isolated from cloned T cells obtained

during the *HPRT* cloning assays (see the later section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes). The polymorphisms and primers used for amplification are listed in Table 8. DNA was isolated from the clones using standard methods.

PCR was performed using HotStar *Taq* (Qiagen, Valencia CA) and Qiagen's proprietary buffers in a 25-μL reaction mixture. For the *ADH2* polymorphism, reactions contained 1× HotStar buffer, 5 μL Qiagen (Q) buffer, 20 μM dNTPs, 10 pmol each primer, and a final $MgCl_2$ concentration of 3.5 mM. For the *ADH3*, *EH113*, and *EH139* polymorphisms, reactions contained 1× HotStar buffer, 5 μL Q buffer, 20 μM dNTPs, 10 pmol each primer, and a final $MgCl_2$ concentration of 4.5 mM. For the *CYP2E1* 5′ polymorphism, reactions contained 1× HotStar buffer, 20 μM dNTPs, 10 pmol each primer, and a final $MgCl_2$ concentration of 3.5 mM. For the *CYP2E1* intron 6 polymorphism, reactions contained 1× HotStar buffer, 5 μL Q buffer, 20 μM dNTPs, 10 pmol each primer, and a final $MgCl_2$ concentration of 1.5 mM.

PCR conditions included an initial 95°C for 15 minutes to activate the HotStar *Taq*, and then 36 cycles of: 94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 1 minute; and finalized with 72°C for 10 minutes. Annealing was at 62°C instead of 55°C for the *EH139* polymorphism and at 57°C for the *CYP2E1* 5′ polymorphism. Amplification products were digested with the appropriate restriction enzyme (Table 8) for 3 hours according to the manufacturer's recommendations (New England Biolabs, Beverly MA). The products were made visual on an ethidium-stained (25 μg/100 mL gel) 2% agarose gel (except for the *EH113* polymorphism, which was separated on an acrylamide gel because of the small difference in the size of the cleaved and uncleaved product). The products were made visual with UV illumination and a photograph was recorded. The expected fragments for each polymorphism are shown in Table 8.

Table 8. Polymorphisms for Metabolism Genes

Gene	Polymorphism	Restriction Enzyme	Fragment Sizes	Frequency	Forward Primer	Reverse Primer	Reference
ADH2	Arg47	*Tsp*45I uncut	108 bp	0.95	AATCT TTTCT GAATC TGAAC AG	GAAGG GGGGT CACCA GGTTG C	Xu et al 1988
	His47[a]	*Tsp*45I cut	44 bp & 64 bp	0.05			
ADH3	Ile349[b]	*Ssp*I cut	83 bp & 56 bp	0.50	CTTTA AGAGT AAAGA ATCTG TCC	ACCTC TTTCC AGAGC GAAGC AG	Xu et al 1988
	Val349[c]	*Ssp*I uncut	140 bp	0.50			
EH	Tyr113	*Pfl*FI uncut	231 bp	0.60	CTTGA GCTCT GTCCT TCCCA TCGC	AATCT TAGTC TTGAA GTGAC GGT	Lancaster et al 1996
	His113[d]	*Pfl*FI cut	209 bp & 22 bp	0.40			
EH	His139	*Rsa*I cut	174 bp, 21 bp & 62 bp	0.22	GGGGT ACCAG AGCCT GACCG T	AACAC CGGGC CCACC CTTGG C	Hassett et al 1994
	Arg139[e]	*Rsa*I uncut	295 bp & 62 bp	0.78			
CYP2E1	*C1* (5′)	*Rsa*I cut	350 bp & 60 bp	0.98	CCAGT CGAGT CTACA TTGTC A	TTCAT TCTGT CTTCT AACTG G	Le Marchand et al 1998
	C2 (5′)	*Rsa*I uncut	410 bp	0.02			
CYP2E1	*C* (intron 6)	*Dra*I uncut	686 bp	0.29	CTGCT GCTAA TGGTC ACTTG	GGAGT TCAAG ACCAG CCTAC	Lin et al 1998
	D (intron 6)	*Dra*I cut	335 bp & 351 bp	0.71			

[a] *ADH2*2* allele = His47 instead of Arg47.
[b] *ADH3*1* allele = Arg271 and Ile349.
[c] *ADH3*2* allele = Gln271 and Val349.
[d] *EH*113* allele = His113 instead of Tyr113.
[e] *EH*139* allele = Arg139 instead of His139.

Results: Genotypes

We determined the *GSTM1* and *GSTT1* genotypes for each of the 83 study subjects. Of all workers, 30 (36.1%) were shown to be homozygous for the *GSTM1*-null allele. Assuming a Hardy-Weinberg equilibrium, this calculates to a gene frequency of 0.60 for the null allele. Analyzing the distribution of *GSTM1* genotypes over the exposure groups showed that 9 of the 25 (36.0%) administrative control subjects, 10 of the 24 (41.7%) monomer production workers, and 11 of the 34 (32.4%) polymerization workers were homozygous for the null allele. The differences among the exposure groups were not statistically significant (Table 9).

Analysis of the *GSTT1* genotype revealed that 13 of the 83 (15.7%) subjects were homozygous for the null allele. Again assuming Hardy-Weinberg equilibrium, this produces a gene frequency of 0.40 for this null allele. The study-wide distribution of homozygosity for this null allele was 5 of the 25 (20.0%) administrative control subjects, 4 of the 24 (16.7%) monomer production workers, and 4 of the 34 (11.8%) polymerization workers. These exposure group differences were not statistically significant (Table 9).

The distributions of genotypes for *CYP2E1*, *EH*, and *ADH* are given in Table 9. Genotypes for these enzymes could be determined only for the 77 subjects whose T lymphocytes were successfully cloned during the *HPRT* assay. Therefore, results were not obtained for two monomer production and four polymerization workers. An additional monomer production worker was not genotyped for the *CYP2E1* intron 6 polymorphism because of technical failure. The distributions of genotypes in the control, monomer, and polymer groups did not differ significantly for any of the *CYP2E1*, *EH*, or *ADH* polymorphisms that we investigated.

The three exposure groups were fairly well balanced with regard to metabolic genotypes and no differences among groups were statistically significant. Although genotype would not therefore bias group comparisons of biomarkers, the effects of genotype, smoking, and other questionnaire data on biomarkers were investigated. These results are given in the chapters pertaining to each biomarker.

Table 9. Genotypes by Group

	Control *n* (%)	Monomer *n* (%)	Polymer *n* (%)
	(*n* = 25)	(*n* = 24)	(*n* = 34)
GSTM1			
Positive	16 (64.0)	14 (58.3)	23 (67.6)
Null	9 (36.0)	10 (41.7)	11 (32.4)
GSTT1			
Positive	20 (80.0)	20 (83.3)	30 (88.2)
Null	5 (20.0)	4 (16.7)	4 (11.8)
	(*n* = 25)	(*n* = 22)[a]	(*n* = 30)[a]
***CYP2E1* 5′ Promoter Region**			
C1/C1	24 (96.0)	21 (95.5)	30 (100.0)
C1/C2	1 (4.0)	1 (4.5)	—
***CYP2E1* Intron 6**			
C/D	3 (12.0)	4 (19.0)[b]	4 (13.3)
D/D	22 (88.0)	17 (81.0)[b]	26 (86.7)
EH*113			
Tyr113/Tyr113	15 (60.0)	6 (27.3)	15 (50.0)
Tyr113/His113	6 (24.0)	12 (54.5)	9 (30.0)
His113/His113	4 (16.0)	4 (18.2)	6 (20.0)
EH*139			
Arg139/Arg139	2 (8.0)	3 (13.6)	1 (3.3)
Arg139/His139	11 (44.0)	2 (9.1)	10 (33.3)
His139/His139	12 (48.0)	17 (77.3)	19 (63.3)
ADH2			
Arg47/Arg47	23 (92.0)	19 (86.4)	28 (93.3)
Arg47/His47	2 (8.0)	3 (13.6)	2 (6.7)
ADH3			
Ile349/Ile349	9 (36.0)	6 (27.3)	7 (23.3)
Ile349/Val349	15 (60.0)	12 (54.5)	15 (50.0)
Val349/Val349	1 (4.0)	4 (18.2)	8 (26.7)

[a] The *CYP2E1*, *EH*, and *ADH* genotypes could be determined only for those subjects whose T lymphocytes were successfully cloned in the *HPRT* cloning assays (see the later section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes). Thus, for these genotypes, only 22 of 24 monomer production workers and 30 of 35 polymerization workers were genotyped.

[b] The samples for 1 monomer production worker were lost due to technical failure; therefore *n* = 21.

MEASUREMENT OF OCCUPATIONAL EXPOSURE TO BD

This section describes the methods used to estimate workers' exposure to BD and their coexposure to the VOCs benzene, toluene, and styrene. Evaluating the usefulness of a biomarker as a measure of BD exposure requires that personal exposures be accurately estimated. This is best accomplished by personal monitors that record BD exposure concentrations over the period of time that is relevant to the formation and persistence of the biomarker. The longitudinal concentration data are then combined to summarize an individual's exposure over the entire time period.

A number of methods have been developed to measure a worker's exposure to BD both to evaluate health hazards and to monitor compliance with safety standards. Such methods have been reviewed by an Analytical Working Group that consists of experts from the Lower Olefins Sector Group of the European Chemical Industry Council (CEFIC) and from the Environmental Health and Product Safety Committee of the International Institute of Synthetic Rubber Producers (IISRP). In a report issued in September 1996, the Working Group identified three solid sorbent methods of analysis to provide high levels of accuracy and precision: NIOSH 1024, 3M3520, and MDHS 63 (Bianchi et al 1996). With the NIOSH 1024 method, a pump is used to draw air through a glass tube packed with a solid sorbent. In the other two methods air is not pumped; instead, BD diffuses from the air onto a sorbent in either a plastic badge (for method 3M3520) or a stainless-steel tube (for method MDHS 63) (Health and Safety Executive [HSE] 1989; DHHS 1994; Minnesota Mining and Manufacturing Corp 1996). In NIOSH 1024 and 3M3520, BD is desorbed with a solvent and in MDHS 63 it is thermally desorbed. In all three methods, BD is analyzed by gas chromatography (GC).

Several factors about laboratories and the samplers themselves contributed to our decision to use the MDHS 63 method of sampler analysis. First, because our study subjects were employed in the Czech Republic and samples would be collected there, it was important to use a European laboratory (which generally do not perform analyses via the NIOSH 1024 method). Second, to maximize accuracy, it was essential to choose a laboratory that routinely conducts a high volume of analytic work and have our samples analyzed using its preferred method.

Thus, we ruled out the NIOSH 1024 method because (1) it would not be used in a European laboratory, and (2) a sampler that employs a pumped tube may feel more intrusive to the subjects (who are unaccustomed to wearing personal samplers) and may reduce participation in the study. Although both the 3M3520 and MDHS 63 methods were judged to be acceptable, we eliminated 3M3520 as an option; BD recovery by this method is sometimes as low as 75% and, although in theory low desorption efficiency will work if it is known and constant, variability still occurs and reduces the accuracy of the results. Furthermore, MDHS 63 has been validated and approved for use in sampling BD by a national or international authority or regulator, whereas 3M3520 has not, even though it has been specifically recommended by its manufacturer to be used for BD sampling (Bianchi et al 1996).

Finally, the government Health and Safety Laboratory of the UK (in Sheffield) met all of our criteria for a laboratory, uses the MDHS 63 method of analysis routinely, and was available to do the work.

EXPOSURE ASSESSMENT PROTOCOL

Exposure is not a single quantity, but is rather a function that describes the fluctuations in exposure intensity over a period of time; thus, the sampling design and the choice of metric to estimate exposure depends on the purpose of the study. Continuously monitoring each worker over the duration of the study would provide the most complete characterization of exposure, but this was not feasible. Instead, we monitored each subject on a sample of workshifts and used those data to estimate exposure over the time period of interest. Because fluctuations in both timing and intensity would affect the biomarkers in this study differently, we designed a protocol that would allow for both short-term and intermediate-term exposure to be monitored.

Exposure Measurements from Personal Monitors

The protocol specified that each exposed subject be personally monitored for BD over his entire workshift (usually 8 hours) on 10 workdays during a 60-day exposure assessment period. The sampler measured the cumulative amount of BD the subject was exposed to during the time the device was worn. We divided this quantity by the duration of the monitoring period to yield the TWA BD concentration for the subject's workshift.

We planned for three of the BD measurements to be made on the last 3 workshifts of the 60-day assessment period to coincide with collecting urine samples to assess biomarkers that would reflect recent (short-term) exposure (eg, urinary metabolites). The remaining seven measurements were to be made at irregular intervals during the 60-day assessment period, with the first one scheduled at the beginning. These seven semi-random measurements, together with the three measurements made at the end of

the period, would be used to summarize each subject's exposure over the 60-day period to assess biomarkers that would reflect intermediate-term exposure (eg, Hb adducts and genetic effects). The 25 control subjects were to be considered a homogeneous, unexposed group, and each worker would have one 8-hour BD measurement on one workshift during the assessment period.

To assess concurrent exposure to the VOCs benzene, styrene, and toluene, six sets of seven measurements each would be made at irregular intervals that would cover all shifts for six exposed groups of workers (a total of 42 measurements). Seven additional measurements were to be made at irregular intervals to cover all shifts of the control subjects. The protocol for these VOC measurements was designed to determine if any coexposures might track with BD and confound the interpretation of exposure results. A more extensive assessment of VOCs was not thought to be warranted because of the cost, the availability of personal monitors, and the fact that VOCs were not the primary focus of this study. (We had also planned to measure exposure to dimethyldithiocarbamate, but we learned that this compound is not used at the facilities participating in the study and therefore these measurements were not made.)

Availability of the sorbent tubes used for monitoring BD and VOC exposure precluded strict adherence to our exposure assessment protocol. It was not possible to fully implement the semi-random sampling plan, but all exposed subjects had BD exposure measurements near the beginning, middle, and end of their exposure assessment period. The actual timing of both the BD and VOC exposure measurements are given for each subject later in the Results section.

Workplace Area Measurements

In addition to personal monitoring for TWA BD and concurrent VOC exposures, we took workplace area measurements of BD and VOCs. The exposure assessment protocol initially included a plan to measure time-intensity patterns of BD exposure (peaks) using a mini photoionization detector connected to a data logger. However, because of a defect in the apparatus (and because of concerns expressed by the company participating in the study), this method was not used. Instead, workplace area BD levels were measured as 15-minute concentrations using pumped samples from selected locations (18 in administration, 60 in the monomer production unit, and 89 in the polymerization unit; total of 167 measurements). Concentrations from 15 minutes of sampling (rather than from a full workshift, for example) were deemed sufficient to evaluate workplace area levels of the agents of interest because these measurements were made primarily to assess whether the personal monitoring results (recorded as workshift TWAs) were "reasonable" as determinants of the workers' exposure.

Workplace area concentrations of benzene, styrene, and toluene were also measured in the same manner (12 in administration, 11 in the monomer production unit, and 14 in the polymerization unit; total of 37 measurements).

EXPOSURE SAMPLING AND ANALYSIS

BD: Materials and Methods

In the MDHS 63 method used by Sheffield, BD is sampled by exposing a passive diffusive sampler to air for a measured time period (Fields 1986; Hamlin and Saunders 1986; HSE 1989). The sampling rate is determined by calibrating the equipment in a standard atmosphere before use. BD gas migrates down the tube by diffusion and is sorbed on the molecular sieve. The collected gas is desorbed by heat and is transferred under inert carrier gas into a gas chromatograph equipped with a flame ionization detector (FID), where it is analyzed. This method was developed to determine TWA concentrations of BD gas in workplace atmospheres. The method is suitable for sampling over periods that range from 30 minutes to 8 hours and it can be used for both personal and fixed-location monitoring.

The validity of this method for measuring BD exposure has been examined by following a diffusive sampler evaluation protocol (HSE 1983) that uses a PerkinElmer diffusive tube containing a molecular sieve sorbent and a silicone membrane in the diffusion cap (Cox 1985; Cox et al 1987). From laboratory experiments, the uptake rate, or calibration factor, has been found to be 0.59 cm^3/min or 1.30 ng/ppm/min; the random error of the uptake rate is 15%, which is expressed as a coefficient of variation. The uptake rate does not vary significantly with the time or concentration of exposure; high humidity does not adversely affect it; and it is not significantly affected by ambient air movement, provided a minimum of about 2 ft/min (approximately 0.01 m/sec) is maintained. In tests conducted at Sheffield on samplers spiked with either 1.3 μg or 6.7 μg BD and stored at 4°C or 20°C for 14 or 28 days, the mean recovery of BD compared with freshly prepared standards was 89% or higher; neither time nor temperature significantly affected the percentage of recovery. Similar results were obtained on samplers stored at 40°C for 10 days.

In a field evaluation performed by Sheffield apart from this study, paired sets of diffusive samplers and pumped molecular sieve tubes were exposed simultaneously on the lapels of people at work (Cox et al 1987). The results obtained from log-transformed data yielded a correlation coefficient of 0.937 over exposure concentrations ranging

from 0.4 mg/m^3 (0.2 ppm) to 39.8 mg/m^3 (18.0 ppm). In regression analysis, the slope and intercept did not differ significantly from 1 and 0, respectively, which indicates equivalence. The data from the field evaluation were also examined by paired *t* test. For the 24 pairs of observations, the mean ± SD difference was 0.77 ± 3.5 mg/m^3 (0.35 ± 1.6 ppm). The *t* test results indicated that the mean difference was not significantly different from zero and thus there was no systematic difference in the results obtained by the two methods. These findings demonstrated that the results from the diffusive sampler were unbiased compared with the pumped sampler within the random errors associated with the two methods.

Sheffield assessed the precision and accuracy of the MDSH 63 method by spiking 1.3 μg BD, which is equivalent to about 5.5 mg/m^3 (or 2.5 ppm) BD, onto replicate samplers for 400 minutes each. The relative SD among the results of the spiked samplers was 4% using gas syringes and a static, fresh dilution of pure BD as the source. A bias due to incomplete recovery is not detected in this test and in thermal desorption bias is not easy to detect. By plotting recovery from 200°C to 300°C, the laboratory was able to establish that 270°C was an optimal desorption temperature for BD. However, this was not certain proof that the percentage of recovery would meet the criterion of greater than 95% for thermal desorption samplers that is required by European Standard EN 838 (British Standards Institution 1996). An alternative verification was accomplished by spiking a second sorbent that was completely different in character: dual-bed air toxics tubes (Supelco, St Louis MO, USA) consisting of graphitized carbon and a carbon molecular sieve. Spiking BD and *n*-pentane mixtures on the air toxics tubes established agreement with the recovery from molecular sieve 13X within 5%.

Any compound that coelutes with the BD at the operating conditions chosen by the analyst is a potential interferant. Using an FID, coelutions are occasionally possible; but from the chromatograms in this study, it appeared that samples with significant amounts of BD were unaffected by such interferants. Interfering peaks could have some effect at 0.4 mg/m^3 (0.2 ppm) but not at 4.4 mg/m^3 (2 ppm).

Given the testing described thus far, we decided to use diffusive thermal desorption tube samplers (PerkinElmer). These stainless-steel tubes are 89 mm in length, have an outside diameter of 6.34 mm, an inside diameter of 5.00 mm, and a nominal air gap of 1.50 mm (Brown et al 1981). One end of the tube sampler has a machined internal groove into which a 100-mesh stainless-steel screen is fitted. The cylindrical air gap is defined by the distance between the screen and a removable diffusion cap with a 50-μm silicone membrane (PerkinElmer part L407 0208) to exclude water during BD sampling. The effective air gap between the sorbent and the diffusion cap is nominally 1.50 mm. Empty tubes and screens from PerkinElmer were packed with 500 ± 50 mg molecular sieve 13X, 40–60 mesh (Chrompack; Varian, Walton-on-Thames Surrey, UK). The distance from the screen to the open end of the tube was measured to within 0.02 mm and tubes outside the limits of 14.3 ± 0.3 mm were not used. Assuming that variance in the internal diameter was negligible, the variance of the area/length ratio contributed ≤ 1% to the overall sampling variance from all causes.

Before use, the sorbent tubes were conditioned by heating them slowly under inert carrier gas to 300°C for at least 16 hours. Tubes were then analyzed to ensure that the thermal desorption blank was ≤ 50 ng (HSE 1983). The desorption blank of freshly conditioned tubes was about 0.5 ng BD, equivalent to about 0.0018 mg/m^3 (1 ppb) for an 8-hour sample. Sample blanks were prepared by using tubes identical to those used for sampling and subjecting them to the same handling procedure as the sampling tubes. Spiked sorbent tubes were prepared by injecting known aliquots of BD gas onto clean sorbent tubes. Fresh standards were prepared with each batch of samples with at least three standards at each tube loading level (typical tube loading levels for the replicate standards were in the range of 0.1 to 2 μg BD). When the sampling tubes were sent for analysis, Sheffield was blinded to the identity of ten field blanks and they were analyzed together with the sampling tubes used for exposure monitoring; BD levels in the field blanks ranged from 0 to 7 ng.

Two types of end caps were used with these samplers. For sealing the tubes when they were stored, closed metal end caps with polytetrafluoroethylene (PTFE) or Vitron (Dupont Dow Elastomers, Wilmington DE) seals were used. For sampling, a diffusive end cap that allowed the ingress of vapor through a silicone membrane and metal gauze was used on one end of the tube (the size of the opening matches the cross-section of the tube). To collect a sample, the closed end cap was removed and replaced with a diffusion end cap. For personal sampling, the tube was mounted in the worker's breathing zone (eg, on the lapel). At the end of a measured period of exposure, the diffusion end cap was removed and replaced with the closed cap.

To quantify BD exposure, the sorbent tube was placed in a thermal desorption apparatus. The tube was heated to displace the sorbed gases, which were passed to a gas chromatograph by means of a carrier gas stream. Sample blanks and the samples used to determine desorption efficiency were analyzed in the same way. A PerkinElmer model

ATD-400 was used for desorption with the following conditions:

Primary desorb temperature: 270°C
Desorb time: 10 minutes
Desorb flow: 20 mL/min helium
Typical split ratio: 30:1
Peltier-effect cold-trap: 70 mg Tenax GR at −30°C, backflush configuration
Cold-trap desorb temperature: 300°C

The gas chromatograph was a PerkinElmer Autosystem XL with the following specifications:

Column: fused silica 60 m × 0.25 mm VOCOL (Supelco)
Film thickness d_f: 1.5 μm
Carrier gas: helium at 32 psi inlet pressure
Temperature program: 35°C held for 4 minutes, 4°C/min to 60°C, postrun 200°C for 5 minutes (BD approximate retention time 6 minutes)
Detector: Flame ionization
Data acquisition: PerkinElmer Turbochrom v4.2

To determine desorption efficiency, aliquots of BD gas were injected directly into the gas chromatograph and a calibration graph was prepared showing the peak response plotted against the analyte injected. Then the spiked tube standards were analyzed and a second calibration graph was prepared. The response of a tube standard was within 95% of that of the corresponding gas standard injected directly.

VOCs: Materials and Methods

The methods used by Sheffield to measure exposures to benzene, toluene, and styrene by thermally desorbed diffusive passive samplers were essentially the same as those for BD. PerkinElmer diffusive tube validations for benzene, toluene, and styrene followed the generic method MDHS 80 (HSE 1995). Benzene and styrene were fully validated by protocol EN 838 (British Standards Institute 1996). Toluene was partially validated, but its assessment included field comparisons with a reference source.

The methods for benzene, toluene, and styrene measurement met the following precision and accuracy requirements: desorption efficiency was greater than 98%; recovery after 2 weeks at ambient temperature was greater than 98%; and expanded uncertainty of the final result (sampling rate + analysis) within 30% (95% confidence interval [CI]) was up to twice the workplace limit value (for 0.5–8 hours exposure, 20%–80% relative humidity, 10–30°C).

The same PerkinElmer tube sampler used for BD was used for VOC sampling except that a diffusion cap without the silicone membrane was used (PerkinElmer part L407 0207). The tubes were packed with 250 ± 25 mg Chromosorb 106, 60–80 mesh (Chrompack). For thermal desorption, a PerkinElmer model ATD-400 was used with the following conditions:

Primary desorb temperature: 230°C
Desorb time: 10 minutes
Desorb flow: 50 mL/min helium
Typical split ratio: 30:1
Peltier-effect cold-trap: 30 mg Tenax TA at −30°C, forward flush configuration
Cold-trap desorb temperature: 300°C

The gas chromatograph was a PerkinElmer Autosystem with the following specifications:

Columns: dual columns (SGE Europe, Milton Keynes UK) fitted in parallel from the thermal desorber to the transfer line
- Fused silica 50 m × 0.22 mm BP-1; film thickness d_f: 1.0 μm
- Fused silica 50 m × 0.22 mm BP-10; film thickness d_f: 0.5 μm

Carrier gas: helium at 46 psi inlet pressure
Temperature program: 50°C to 180°C at 5°C/min
Detectors: Dual flame ionization
Data acquisition: PerkinElmer Turbochrom v4.2

To calibrate the equipment Sheffield used the liquid spiking technique (HSE 1995; Woolfenden and McClenny 1997). Known quantities of benzene, toluene, and styrene were each diluted in methanol and prepared by gravimetry. Aliquots were spiked on calibration tubes packed with Chromosorb 106, and the tubes were analyzed using the same procedure used for the samples. VOC calibrations prepared in this form are traceable to primary standards. European certified reference thermal desorption tubes loaded with benzene, toluene, and xylene (which is the closest chemical to the BD and VOC analyses in question; Certified Reference Material 112) were used as an additional check on the procedure.

Quality Control

Internal quality control (QC) procedures for both BD and VOCs were in accordance with MDHS 71 analytical quality in workplace air monitoring (HSE 1991). For external quality assurance, Sheffield takes part in the United Kingdom's Workplace Analysis Scheme for Proficiency

(WASP) and a separate unit within Sheffield administers WASP. (This is similar to the American Industrial Hygiene Association's Proficiency Analytical Testing scheme in the USA, except that in the Proficiency Analytical Testing scheme, membership involves American Industrial Hygiene Association laboratory accreditation, whereas in the United Kingdom, accreditation is achieved via a separate organization, United Kingdom Accreditation Scheme, that takes note of performance in proficiency schemes, but does not administer them. About 100 laboratories participate in WASP, of which about 75% are in the United Kingdom; most of the rest are in other European countries and a few laboratories are in the United States, Japan, and Australia.) WASP ranks laboratories according to a long-term performance statistic (best performance in four out of the last five quarterly rounds). The long-term performance of Sheffield in thermal desorption of benzene, toluene, and xylene is equivalent to a relative SD of 2% from the reference or "true" value.

Statistical Methods

A complete description of all statistical methods is presented in the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Data Processing and Statistical Analysis.

RESULTS

BD Exposure Measurements from Personal Monitors

Between 19 March 1998 and 12 June 1998, 536 individual workshift BD exposure measurements were obtained

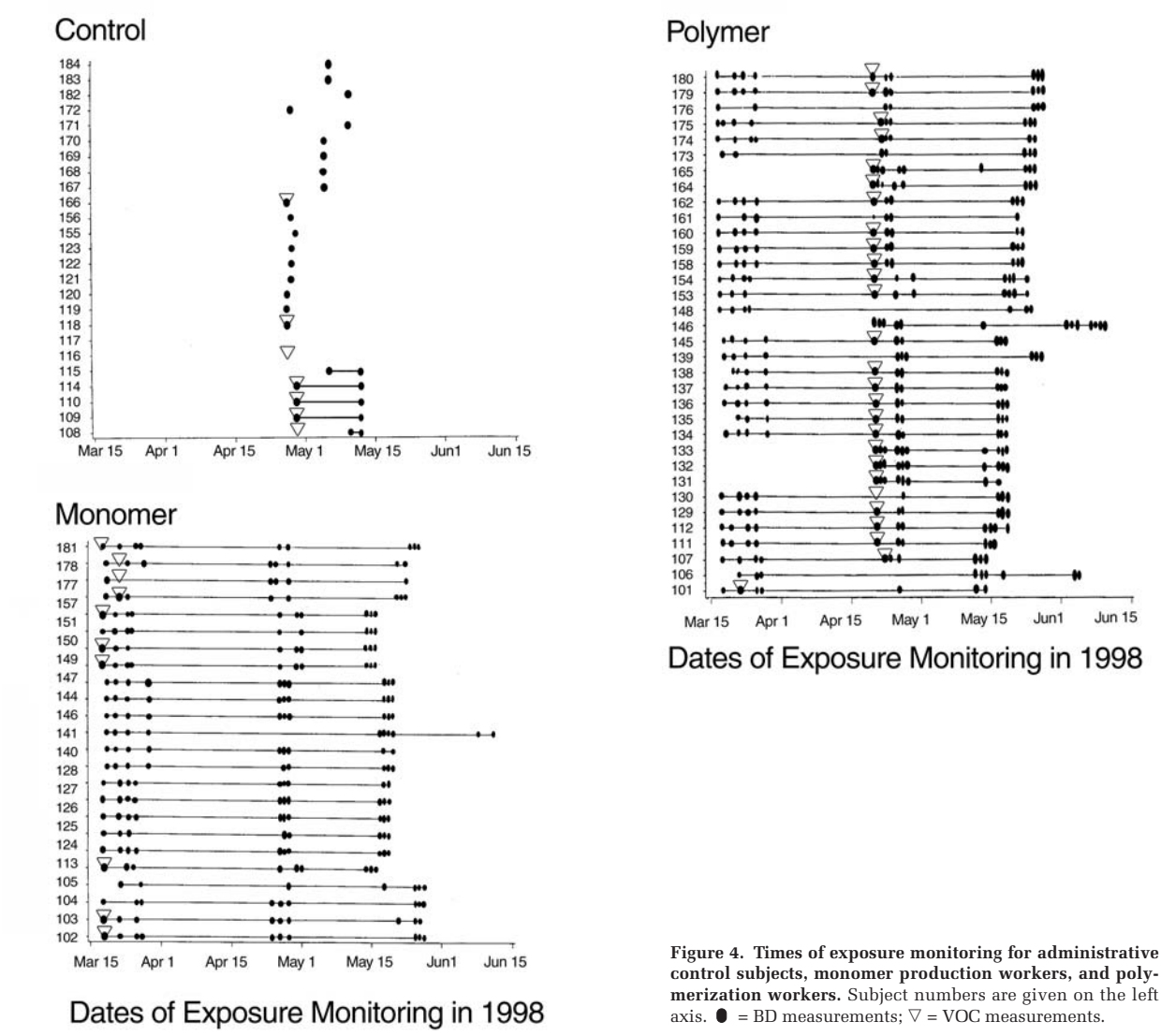

Figure 4. Times of exposure monitoring for administrative control subjects, monomer production workers, and polymerization workers. Subject numbers are given on the left axis. ● = BD measurements; ▽ = VOC measurements.

for the exposed workers using diffusive solid sorbent tubes: 217 measurements for the 24 BD monomer production workers and 319 measurements for the 34 BD polymerization workers (each measurement was recorded as the TWA for the subject's workshift). Some workers had less than the scheduled 10 measurements due to discrepancies in tube identification numbers recorded in Prague and Sheffield, but most workers had at least eight measurements. In addition, 28 individual workshift BD exposure measurements were made on the 25 control subjects. Figure 4 shows the dates of exposure measurement for each subject; a ▽ marks the days on which concurrent exposures to benzene, toluene, and styrene were measured.

The length of time between a worker's first and last exposure measurement was not always 60 days due to the availability of diffusive solid sorbent tubes. The actual exposure assessment period for individual subjects ranged from 28 to 84 days; but for 48 of the 58 exposed subjects, it ranged from 50 to 70 days. The time over which all exposure assessments were made for an individual is hereafter referred to as "over the entire exposure assessment period". Each worker had several measurements made during the interim 70 to 50 days before biological samples were obtained; hereafter, this subset of measurements is referred to as "the exposure interval 70 to 50 days before blood (or urine) sampling". As planned, most subjects had measurements at the beginning, middle, and end of their assessment periods.

The measurements of BD exposure levels, both in the monomer production and in the polymerization facilities, were highly variable. The distributions of individual BD concentrations from the monomer production and polymerization workers, plotted in Figure 5 on a logarithmic scale, show this variability. The lowest values in each histogram correspond to exposures below the LOD (0.0036 mg/m^3 [0.0016 ppm] for an 8-hour exposure monitoring period, but this varied depending on the length of the workshift during which the sorbent tube was worn). Although the values above the LOD appear to approximate a lognormal distribution, more values are below the LOD than would be expected with this distribution (Singh et al 1997). The data are better described by the delta distribution, in which nonzero values are lognormally distributed but a proportion of values are essentially zero (Owen and DeRouen 1980).

Descriptive statistics for the individual BD exposure measurements are presented in Table 10 for each group of workers. For all statistics except the maximum, percentiles, and mean of the delta distribution, a value of 0.0018 mg/m^3 (half the LOD) was used for undetectable exposures. Of all the BD exposure measurements, 8 for administrative

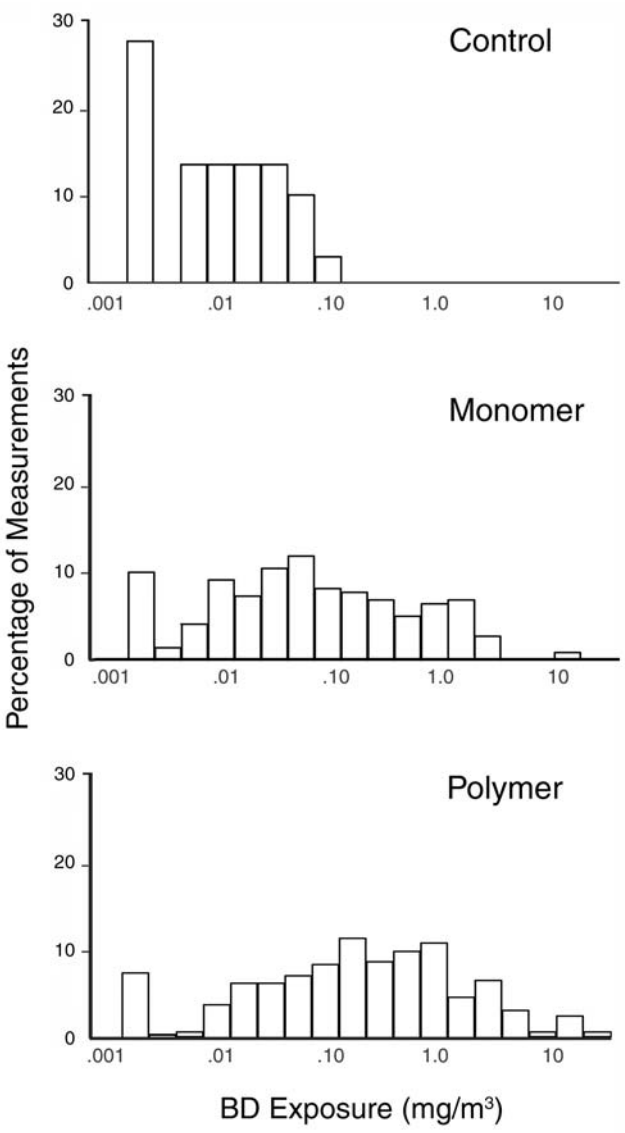

Figure 5. Individual BD exposure measurements for various workshifts by group. Administrative control subjects: 28 measurements; monomer production workers: 217 measurements; and polymerization workers: 319 measurements. BD concentrations are shown on a log scale.

control subjects, 22 for monomer production workers, and 24 for polymerization workers were below the LOD. The mean ± SD BD concentration to which the monomer production workers were exposed, calculated from the 217 individual TWA measurements, was 0.643 ± 2.056 mg/m^3 (0.291 ± 0.930 ppm). The exposures of the polymerization workers were greater, with a mean ± SD concentration of 1.760 ± 4.692 mg/m^3 (0.796 ± 2.123 ppm) calculated from the 319 individual TWA measurements. The control subjects were exposed to quite low BD concentrations, with a

Table 10. Descriptive Statistics for Individual BD Exposure Measurements by Group[a]

	Control	Monomer	Polymer
n Measurements	28	217	319
Mean concentration[b]	0.026	0.643[c]	1.760[c,d]
SD	0.030	2.056	4.692
Minimum	0.002	0.002	0.002
Maximum	0.125	19.909	39.030
50th percentile	0.013	0.074	0.293
90th percentile	0.071	1.886	4.344
Mean of natural log	−4.446	−2.482	−1.422
SD of natural log	1.421	2.194	2.312
Mean of delta distribution	0.026	0.649	2.032

[a] BD exposure was measured in mg/m^3. Each individual exposure measurement was recorded as a TWA for the subject's workshift.

[b] Mean of all workshift TWAs for each group.

[c] Significantly different from control group at $P < 0.05$ by Kruskal-Wallis test.

[d] Significantly different from monomer group at $P < 0.05$ by Kruskal-Wallis test.

mean ± SD of 0.026 ± 0.030 mg/m^3 (0.012 ± 0.014 ppm) calculated from the 28 individual TWA measurements. The monomer production and the polymerization workers' BD exposure levels were significantly different from each other and significantly greater than exposure levels in the administrative control subjects by the nonparametric Kruskal-Wallis test ($P < 0.001$).

Consistent with the results of the Kruskal-Wallis test, the other measures of central tendency in Table 10 show the same pattern of increasing exposure for the control, monomer, and polymer groups. The median values (50th percentile) were much lower than the means, reflecting the skewness of the data distributions. The estimated mean of the delta distribution for each group was very similar to the arithmetic mean. Group differences in the means of the natural logarithms of BD concentrations were tested by ANOVA, with subject included as a random effect. Unlike the nonparametric Kruskal-Wallis test for differences in means, which assumes that all measurements are independent, ANOVA adjusts for correlations between measurements made on the same subject. However, the results from both analyses were the same; all three groups differed significantly from each other.

The individual TWA measurements from all monomer production workers ranged from 0.0018 mg/m^3 (undetectable) to 19.909 mg/m^3. For workers in the polymerization facility, the range of TWA concentrations was even broader: from 0.002 to 39.030 mg/m^3. In both the monomer production and polymerization facilities, most of the variability was due to a few high values, given that 90% of the measurements for the monomer production workers were less than 1.883 mg/m^3 and 90% of the measurements for the polymerization workers were below 3.985 mg/m^3. By contrast, TWA exposure concentrations for administrative control subjects covered a narrow range from 0.002 to 0.125 mg/m^3.

ANOVA was conducted on components of the data to determine the sources of variation in the exposure measurement data for the monomer production and polymerization workers. For these analyses, exposure concentration was expressed as the natural logarithm of the TWA concentration and both date of exposure monitoring and subject were modeled as additive random effects. The results confirm that the main source of variability in the measurement data was day-to-day variation within subject. For the monomer production workers, between-subject variability (1.058) and between-day variability (0.769) were both considerably smaller than the residual variability of 2.876. For the polymerization workers, the between-subject variability (0.723) was twice that of the between-day variability (0.360), but the residual variability (4.150) was almost four times as large as both of these combined. Individual BD concentrations for subjects by day of exposure monitoring are displayed in Figure 6. Mean values for each day are indicated by a horizontal line and do not exhibit a temporal trend.

Summary Metrics for BD Exposure from Personal Monitors

A primary goal of personal exposure monitoring was to provide for each monomer production and polymerization worker a summary measure of the exposure he received over the 60-day assessment period that could be correlated to biomarkers of exposure. Not enough exposure measurements were obtained for each worker to determine a distribution for each worker. However, the minimal, maximal, and median values for each worker (shown in Table 11 along with the results of the variance component analysis described above) suggest that each worker's daily exposures may follow a delta distribution. The mean of the delta distribution was therefore considered as a potential metric for summarizing each worker's exposure. Other metrics that were considered included the arithmetic mean, the geometric mean, the mean of the lognormal distribution, and the maximal value.

Computation of all metrics except the mean of the delta distribution and the maximal value required that some value be imputed for measurements below detection. Because the LOD was extremely low (0.0036 mg/m^3), the

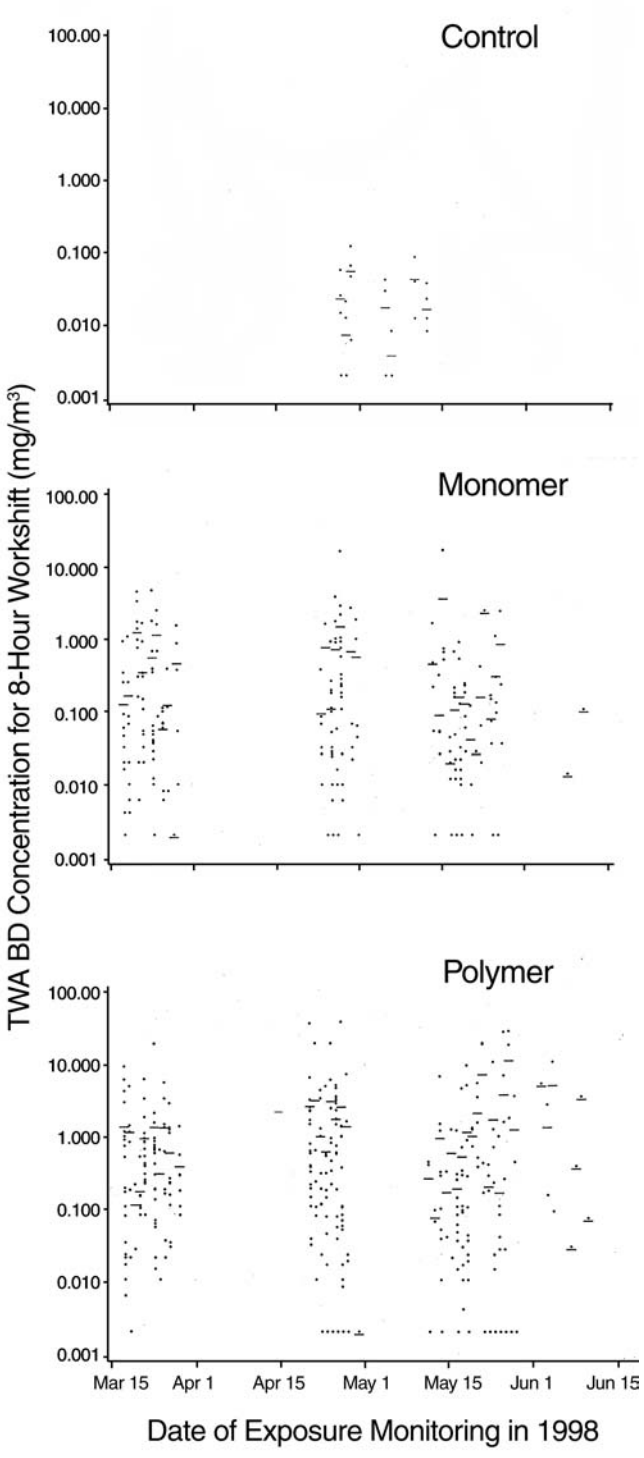

Figure 6. Measurements of BD presented on a log scale by date of monitoring for each group of workers. • = 8-hour TWA BD concentrations; — = daily mean concentrations.

choice of value for imputation had little effect on the arithmetic mean. For example, average exposures for monomer production and polymerization workers with one or more undetectable exposures varied in the fourth decimal point or less when zero, half the LOD, or the LOD was substituted for these exposures. The imputed value had a larger effect on the mean of the lognormal distribution, which is based on both the mean and variance of the logarithm of BD concentration. Because values close to zero have large, negative logarithms, a small change in the imputed value could have a substantial effect on the variance estimate and hence the estimated mean of the lognormal distribution.

The various exposure metrics computed for each monomer production and polymerization worker are given in Table 11. Although using the mean of the delta distribution has the advantage of not requiring imputation of values below the LOD, it has several drawbacks. Its computation is based on estimates of the proportion of values below the LOD and the mean and variance of the logarithm of values above the LOD, all of which are imprecise because most subjects have 10 or fewer measurements. Lack of stability is also an issue, as it is for the estimated mean of the lognormal distribution, and both methods can lead to estimates that are not very meaningful. For example, the means of the lognormal and delta distributions for subject 101 in the polymer group are much larger than the maximal value observed for this worker. Such results may also occur when the lognormal or delta distributions are not appropriate representations of the empirical distributions.

The arithmetic mean, on the other hand, is a valid descriptive measure regardless of the data distribution. In addition, the arithmetic mean reflects cumulative exposure over the study period, which may be the most relevant exposure parameter for adduct production and for some of the biomarkers of effect being evaluated in this study. In light of these advantages, and because it is stable when different values must be imputed for undetectable exposures, the arithmetic mean was selected as the primary exposure metric for subsequent correlation analyses with biomarker data. However, when used to rank subjects' exposures, all of the metrics were highly correlated (Table 12) and because most statistical analyses were based on nonparametric methods, very similar results were obtained regardless of the metric used.

Exposure data for workers in the control group (most of whom had only one exposure measurement) are also presented in Table 11. In designing the study, we assumed that all control subjects would have similar, low-level exposures and that the average of all measurements for the group would provide a good estimate of each worker's exposure. Although this appears to be the case, using the

Table 11. Metrics Summarizing BD Exposure for Each Subject[a]

Subject	Number of Workshift TWAs	Minimum (mg/m^3)	Maximum (mg/m^3)	Median (50th Percentile)	Mean	Geometric Mean	Lognormal Distribution	Delta Distribution
Administrative Control Subjects								
108	2	0.009	0.090	.	0.050	0.028	.	.
109	2	0.009	0.048	.	0.029	0.021	.	.
110	2	0.007	0.013	.	0.010	0.009	.	.
114	2	0.040	0.125	.	0.083	0.070	.	.
115	2	0.002	0.024	.	0.013	0.007	.	.
116	NO[b]	0.018	0.029	.	0.023	0.021	.	.
117	NO	0.018	0.029	.	0.023	0.021	.	.
118	1	0.015	0.015	.	0.015	0.015	.	.
119	1	0.059	0.059	.	0.059	0.059	.	.
120	1	0.026	0.026	.	0.026	0.026	.	.
121	1	0.002	0.002	.	0.002	0.002	.	.
122	1	0.022	0.022	.	0.022	0.022	.	.
123	1	0.002	0.002	.	0.002	0.002	.	.
155	1	0.068	0.068	.	0.068	0.068	.	.
156	1	0.002	0.002	.	0.002	0.002	.	.
166	1	0.002	0.002	.	0.002	0.002	.	.
167	1	0.002	0.002	.	0.002	0.002	.	.
168	1	0.002	0.002	.	0.002	0.002	.	.
169	1	0.031	0.031	.	0.031	0.031	.	.
170	1	0.044	0.044	.	0.044	0.044	.	.
171	1	0.042	0.042	.	0.042	0.042	.	.
172	1	0.013	0.013	.	0.013	0.013	.	.
182	1	0.013	0.013	.	0.013	0.013	.	.
183	1	0.002	0.002	.	0.002	0.002	.	.
184	1	0.009	0.009	.	0.009	0.009	.	.
Arithmetic Mean		0.018	0.029	.	0.023	0.021	.	.
SD		0.019	0.031	.	0.023	0.021	.	.
Monomer Production Workers								
102	10	0.002	0.257	0.015	0.062	0.020	0.081	0.071
103	9	0.002	1.252	0.183	0.398	0.163	1.068	0.827
104	9	0.004	0.332	0.073	0.093	0.050	0.132	0.122
105	7	0.040	2.803	0.348	0.674	0.322	0.791	0.728
113	9	0.002	0.779	0.073	0.243	0.062	0.809	0.523
124	10	0.002	0.860	0.055	0.161	0.056	0.238	0.205
125	8	0.002	1.058	0.029	0.195	0.028	0.399	0.241
126	10	0.013	1.030	0.119	0.238	0.109	0.269	0.252
127	10	0.018	3.388	0.064	0.396	0.071	0.240	0.215
128	9	0.002	2.059	0.073	0.411	0.093	0.820	0.591
140	9	0.002	0.246	0.018	0.053	0.022	0.064	0.058
141	9	0.002	1.135	0.018	0.146	0.024	0.115	0.096
142	10	0.011	3.991	0.191	0.694	0.155	1.247	0.939
143	10	0.002	2.556	0.330	0.689	0.152	3.556	2.002
144	10	0.035	5.366	1.540	1.861	0.869	3.188	2.820
147	10	0.002	0.315	0.018	0.072	0.019	0.102	0.084
149	10	0.002	0.838	0.029	0.174	0.037	0.349	0.253

(Table continues next page)

[a] BD exposure was measured in mg/m^3. Each individual exposure measurement was recorded as a TWA for the subject's workshift.

[b] NO = Exposure measurements were not obtained for these subjects and they were assigned the mean values for the group.

Table 11 *(Continued)*. Metrics Summarizing BD Exposure for Each Subject[a]

Subject	Number of Workshift TWAs	Minimum (mg/m^3)	Maximum (mg/m^3)	Median (50th percentile)	Mean	Geometric Mean	Lognormal Distribution	Delta Distribution
Monomer Production Workers *(Continued)*								
150	9	0.002	0.585	0.055	0.165	0.062	0.290	0.242
151	9	0.011	2.970	1.155	1.266	0.559	3.412	2.687
152	10	0.029	19.910	1.489	3.516	1.063	6.131	4.973
157	8	0.002	1.956	0.037	0.416	0.055	0.829	0.495
177	5	0.029	1.896	0.367	0.607	0.304	0.954	0.813
178	8	0.002	19.532	0.084	2.530	0.049	9.908	2.170
181	9	0.002	2.851	0.018	0.348	0.025	0.366	0.228
Arithmetic Mean		0.009	3.249	0.266	0.642	0.182	1.473	0.901
SD		0.012	5.240	0.453	0.854	0.274	2.332	1.201
Polymerization Workers								
101	7	0.002	6.765	2.937	2.421	0.402	58.406	13.472
106	9	0.037	10.619	0.156	1.503	0.310	1.385	1.167
107	10	0.068	4.415	0.411	1.119	0.469	1.317	1.215
111	10	0.029	3.247	0.550	0.838	0.322	1.427	1.220
112	11	0.002	1.762	0.330	0.641	0.175	2.832	1.825
129	10	0.062	39.030	1.602	7.408	1.712	13.073	9.963
130	8	0.011	0.689	0.084	0.188	0.079	0.243	0.217
131	8	0.002	2.317	0.185	0.470	0.131	1.269	0.865
132	10	0.002	2.317	0.031	0.326	0.035	0.513	0.332
133	10	0.011	4.715	0.147	1.154	0.173	2.457	1.597
134	10	0.002	1.177	0.167	0.286	0.111	0.603	0.495
135	9	0.002	3.155	0.724	1.045	0.384	4.724	3.105
136	10	0.011	2.893	0.196	0.657	0.194	1.002	0.832
137	10	0.002	0.587	0.079	0.144	0.070	0.242	0.216
138	10	0.002	1.208	0.156	0.262	0.091	0.689	0.526
139	10	0.002	7.341	0.020	1.076	0.045	10.787	2.589
145	10	0.020	1.740	0.099	0.251	0.091	0.213	0.201
146	13	0.031	5.243	0.385	1.211	0.448	1.623	1.471
148	7	0.011	0.726	0.183	0.336	0.167	0.578	0.499
153	10	0.002	9.530	0.266	1.754	0.208	7.465	3.664
154	11	0.002	6.613	0.660	1.903	0.368	12.882	6.636
158	10	0.002	0.801	0.191	0.232	0.096	0.527	0.432
159	10	0.167	4.343	0.587	0.990	0.590	1.003	0.980
160	9	0.002	19.362	0.684	3.650	0.726	25.765	12.123
161	7	0.018	2.570	0.744	1.206	0.430	3.651	2.506
162	10	0.002	18.718	0.414	2.335	0.371	5.503	3.535
164	8	0.029	37.376	2.442	9.244	1.835	46.019	23.220
165	9	0.011	0.117	0.022	0.040	0.029	0.040	0.040
173	7	0.002	0.405	0.191	0.173	0.051	0.884	0.482
174	9	0.002	6.365	1.014	2.088	0.739	13.458	7.879
175	10	0.002	19.659	2.446	4.669	1.211	45.750	22.076
176	7	0.002	27.562	1.179	4.733	0.653	45.152	14.417
179	10	0.002	28.228	0.317	3.792	0.262	14.864	6.236
180	10	0.110	18.229	0.781	2.848	0.918	2.949	2.666
Arithmetic Mean		0.019	8.818	0.599	1.794	0.409	9.685	4.374
SD		0.035	10.745	0.731	2.108	0.443	15.715	6.102

[a] BD exposure was measured in mg/m^3. Each individual exposure measurement was recorded as a TWA for the subject's workshift.

[b] NO = Exposure measurements were not obtained for these subjects and they were assigned the mean values for the group.

Table 12. Spearman Correlations Between Metrics for Summarizing BD Exposure[a]

	Arithmetic Mean	Geometric Mean	Mean of Lognormal Distribution	Mean of Delta Distribution
Maximum	0.964	0.744	0.872	0.877
Arithmetic mean		0.843	0.942	0.955
Geometric mean			0.775	0.843
Mean of lognormal distribution				0.988

[a] Correlations were based on data from the 58 (24 monomer production and 34 polymerization) exposed workers. All correlations were significantly different from zero at $P < 0.001$.

same exposure value for each worker is problematic in statistical analyses based on individual subjects rather than groups because it ignores the variability in exposure measurements. Average exposures for control subjects were therefore based on the one or two measurements made for each worker. For seven of the workers, the single exposure measurement was below the LOD and a value of 0.0018 mg/m^3 (half the LOD) was used. This had no effect on the nonparametric statistical analyses because these workers were always ranked as having the lowest exposure. Two workers had no usable BD exposure measurement due to discrepancies in sorbent tube numbers and they were assigned the mean of the average exposures of the other control subjects.

As seen in Table 11, the mean of the average exposures computed for each subject are slightly different from the values given in Table 10 because not all subjects had the same number of measurements. The mean of the average BD concentrations for the 25 administrative control subjects was 0.023 ± 0.023 mg/m^3 (0.010 ± 0.010 ppm), for the 24 monomer production workers was 0.642 ± 0.854 mg/m^3 (0.290 ± 0.386 ppm), and for the 34 polymerization workers was 1.794 ± 2.108 mg/m^3 (0.812 ± 0.954 ppm) (see Table 11). All group means were significantly different from each other (Kruskal-Wallis test; $P < 0.001$). The large standard deviations reflect the variability in average 60-day exposures among workers in the same group. The distributions of average exposure concentrations for the workers in each group are shown in Figure 7 on a logarithmic scale. Because average values for most administrative control subjects were based on one measurement, the distribution for the control group is nearly the same as in Figure 5, where the separate exposure measurements were plotted. The average exposures for the monomer production and polymerization workers have approximately lognormal distributions. None of these workers had undetectable BD levels for all of their exposure measurements, so none had average exposures below the LOD.

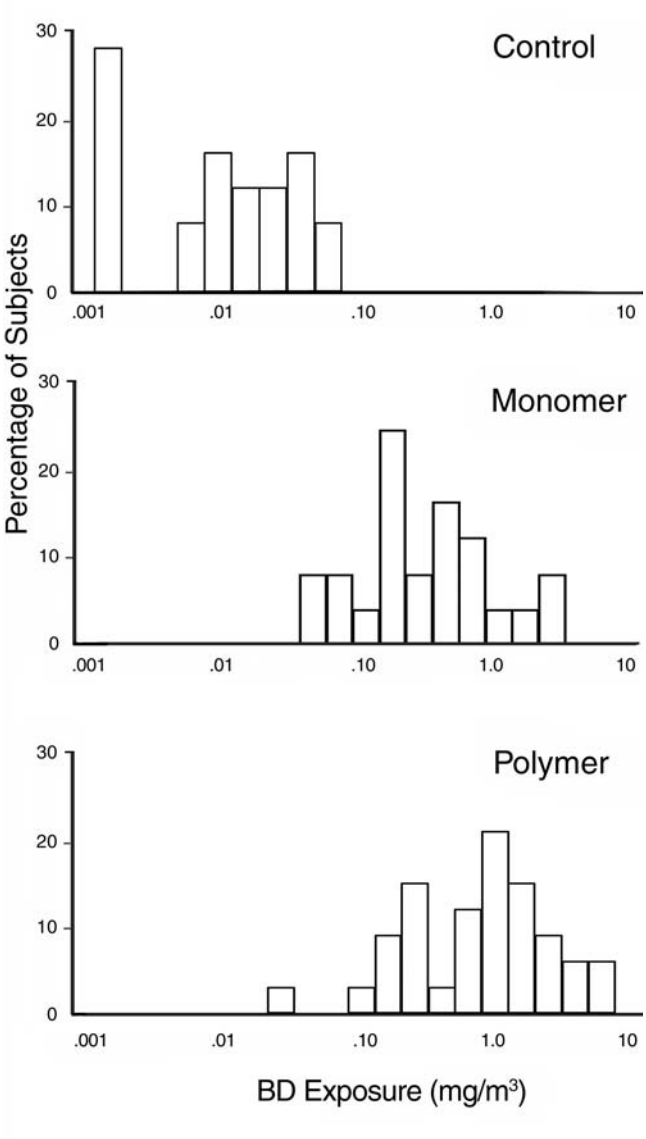

Figure 7. BD exposure for each group averaged over all days of monitoring. Administrative control subjects: $n = 25$; monomer production workers: $n = 24$; and polymerization workers: $n = 34$. BD concentrations are shown on a log scale.

Workplace Area Measurements of BD

The different BD concentrations experienced by workers in the different facilities, as measured by personal monitoring, were also reflected in the 167 workplace area

measurements that were obtained (18 in the control administration unit, 60 in the monomer production unit, and 89 in the polymerization unit). The LOD for these measurements was 0.0005 mg/m^3 (0.0002 ppm) and 18 values were undetectable: 5 in the control administration unit, 4 in the monomer production unit, and 9 in the polymerization unit. Descriptive statistics for the workplace area BD measurements are shown in Table 13; undetectable levels were given a value of 0.00025 mg/m^3 (half the LOD) for computing all statistics except the maximal value, percentiles, and mean of the delta distribution. The monomer production and polymerization units had mean concentrations of 0.316 ± 0.388 mg/m^3 (0.146 ± 0.176 ppm) and 0.892 ± 1.223 mg/m^3 (0.404 ± 0.553 ppm), respectively. In the control administration unit, 18 workplace area measurements showed a low-level mean concentration of 0.043 ± 0.098 mg/m^3 (0.019 ± 0.044 ppm). The means for all three areas were significantly different (Kruskal-Wallis test; $P < 0.001$). The distributions of workplace area BD concentrations for the control administration, monomer production, and polymerization units are shown in Figure 8 on a logarithmic scale and, like the personal exposure measurements, more values are near zero than would be expected with a lognormal distribution.

The mean workplace area concentrations of BD that we measured during the spring of 1998 are somewhat lower than the mean concentrations measured nonrandomly by the company during the preceding 5 years; the ranges of values for area measurements over those years include the average concentrations we recorded (Table 14). The area measurements made by the company during the spring months of 1993 through 1997 were roughly similar to those made during other months of those years, with most discrepancies in the means being due to one or two very high measurements. However, in 1993 and 1996, the mean BD concentrations in the monomer production unit were significantly higher in the spring months than during the rest of the year and, in 1997, the mean BD concentration in the polymerization unit was significantly lower during the spring months (Kruskal-Wallis test, $P < 0.050$).

Table 13. Descriptive Statistics for Workplace Area Measurements of BD[a]

	Administration	Monomer Unit	Polymer Unit
n Measurements	18	60	89
Mean concentration	0.043	0.316[b]	0.892[b,c]
SD	0.098	0.388	1.223
Minimum	0.00025	0.00025	0.00025
Maximum	0.391	1.824	6.241
50th percentile	0.005	0.153	0.414
90th percentile	0.183	0.989	2.400
Mean of natural log	−5.269	−2.272	−1.773
SD of natural log	2.350	2.226	2.850
Mean of delta distribution	0.039	0.500	1.898

[a] BD concentrations were measured in mg/m^3.

[b] Significantly different from administration (control) at $P < 0.05$ by Kruskal-Wallis test.

[c] Significantly different from monomer unit at $P < 0.05$ by Kruskal-Wallis test.

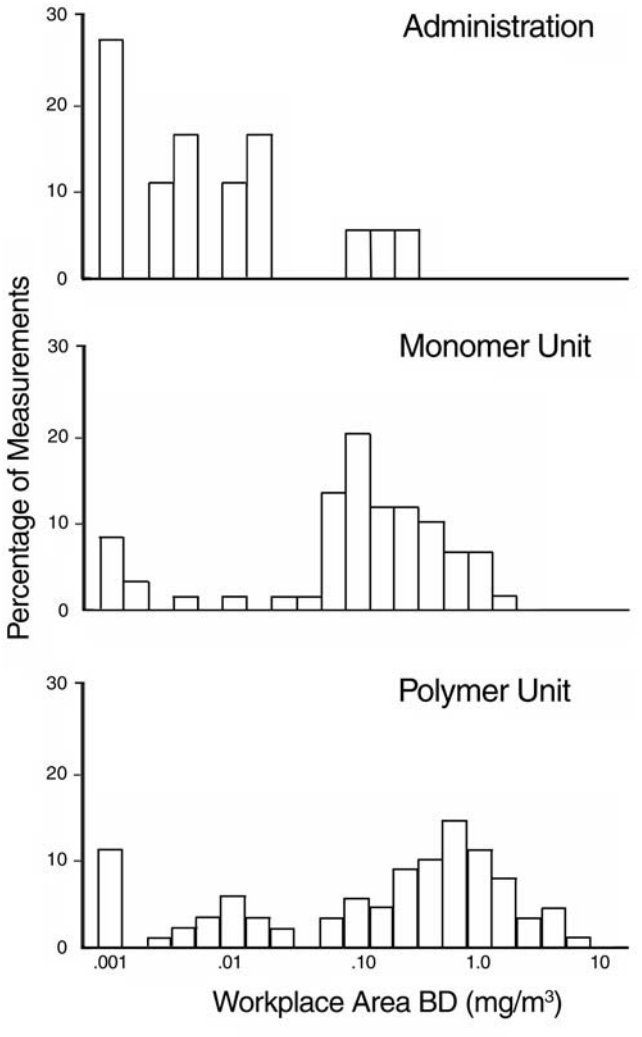

Figure 8. BD measurements in each workplace area. Administration: 18 measurements; monomer production facility: 60 measurements; and polymerization facility: 89 measurements. BD concentrations are shown on a log scale.

Table 14. Yearly Averages of Nonrandom Workplace Area BD Measurements Made by the Company 1993–1997[a]

	All Months			April, May, June		
Year	*n* Measurements	Mean	Range	*n* Measurements	Mean	Range
Monomer Unit						
1993	172	1.512	0–12	48	2.271	0–10
1994	143	0.692	0–10	36	0.778	0–6
1995	158	2.975	0–300	50	7.240	0–300
1996	137	0.891	0–10	15	2.933	0–10
1997	165	0.455	0–9	40	0.650	0–9
Polymer Unit						
1993	193	1.269	0–16	57	1.509	0–16
1994	201	0.771	0–20	56	0.964	0–20
1995	202	2.356	0–34	54	1.796	0–21
1996	183	3.934	0–50	20	1.350	0–5
1997	151	4.377	0–50	46	1.630	0–20

[a] BD concentrations were measured in mg/m^3.

Concurrent VOC Exposure Measurements from Personal Monitors

One personal workshift TWA exposure was measured for each of 7 administrative control subjects, 10 monomer production workers, and 27 polymerization workers. These VOC exposure measurements are given in Table 15 and the descriptive statistics for each group are in Table 16.

The mean benzene exposure concentration for the control group was 0.030 ± 0.015 mg/m^3 (0.009 ± 0.005 ppm), compared with 0.039 ± 0.011 mg/m^3 (0.012 ± 0.003 ppm) for the monomer group and 0.064 ± 0.118 mg/m^3 (0.020 ± 0.037 ppm) for the polymer group; differences between these mean concentrations were not statistically significant. For toluene, the mean exposure concentrations were 0.295 ± 0.280 mg/m^3 (0.078 ± 0.074 ppm), 0.162 ± 0.125 mg/m^3 (0.043 ± 0.033 ppm), and 0.681 ± 1.241 mg/m^3 (0.181 ± 0.329 ppm) for the same groups, respectively; likewise, these concentrations were also not significantly different. For styrene, the mean exposure concentrations were 0.118 ± 0.046 mg/m^3 (0.028 ± 0.011 ppm), 0.147 ± 0.087 mg/m^3 (0.035 ± 0.020 ppm), and 2.121 ± 2.202 mg/m^3 (0.498 ± 0.517 ppm) for the same groups, respectively; this last value was significantly greater than the mean values for both other groups (Kruskal-Wallis test, $P < 0.001$).

Except for five subjects (two administrative control subjects, two monomer production workers, and one polymerization worker), a personal BD measurement was obtained

Table 15. VOC Exposure Measurements for Those Subjects Selected for Personal Monitoring[a]

Subject	Benzene	Toluene	Styrene
Administrative Control Subjects			
108	0.028	0.309	0.216
109	0.025	0.545	0.077
110	0.022	0.011	0.107
114	0.027	0.671	0.089
116	0.058	0.487	0.099
118	0.038	0.027	0.112
166	0.011	0.016	0.127
Monomer Production Workers			
102	0.040	0.145	0.131
103	0.035	0.305	0.078
113	0.052	0.067	0.178
149	0.035	0.083	0.087
150	0.035	0.157	0.112
152	0.062	0.157	0.131
157	0.040	0.452	0.380
177	0.035	0.119	0.103
178	0.024	0.082	0.156
181	0.035	0.053	0.110
Polymerization Workers			
101	0.053	0.043	0.288
107	0.047	0.043	0.504
111	0.040	0.072	2.027
112	0.035	0.038	1.211
129	0.035	0.093	3.136
130	0.032	0.016	3.252
131	0.046	4.678	0.572
132	0.058	4.370	6.224
133	0.058	1.745	0.316
134	0.020	0.030	0.886
135	0.062	1.123	1.435
136	0.028	0.134	0.481
137	0.034	0.912	0.776
138	0.034	0.506	0.307
145	0.065	0.985	0.405
153	0.024	0.009	0.178
154	0.035	0.028	9.628
158	0.653	0.151	3.631
159	0.036	0.014	3.270
160	0.036	0.018	0.673
162	0.030	0.018	2.945
164	0.046	1.849	3.128
165	0.030	1.303	0.369
174	0.033	0.049	2.886
175	0.042	0.046	3.075
179	0.058	0.050	4.909
180	0.067	0.054	0.763

[a] Exposure to VOCs was measured in mg/m^3 and recorded as a TWA for the subject's workshift. Subjects were selected such that measurements could be made at irregular intervals to cover all shifts for exposed workers and administrative control subjects. Each selected subject had one measurement.

Table 16. Descriptive Statistics for Individual VOC Exposure Measurements by Group[a]

	Control	Monomer	Polymer
n Measurements	7	10	27
Benzene			
Mean	0.030	0.039	0.064
SD	0.015	0.011	0.118
Minimum	0.011	0.024	0.020
Maximum	0.058	0.062	0.653
50th percentile	0.027	0.035	0.036
90th percentile	0.058	0.057	0.065
Toluene			
Mean	0.295	0.162	0.681
SD	0.280	0.125	1.241
Minimum	0.011	0.053	0.009
Maximum	0.671	0.452	4.678
50th percentile	0.309	0.132	0.054
90th percentile	0.671	0.378	1.849
Styrene			
Mean	0.118	0.147	2.121[b]
SD	0.046	0.087	2.202
Minimum	0.077	0.078	0.178
Maximum	0.216	0.380	9.628
50th percentile	0.107	0.121	1.211
90th percentile	0.216	0.279	4.909

[a] All VOCs were measured in mg/m^3 and recorded as TWAs for the subjects' workshifts.

[b] Significantly different from both the control and monomer groups at $P < 0.05$ by Kruskal-Wallis test.

Table 17. Spearman Correlations Between BD, Styrene, Benzene, and Toluene Exposures Measured on the Same Day[a]

	Styrene		Benzene		Toluene	
	r	*P*	*r*	*P*	*r*	*P*
BD	0.710	<0.001	0.320	0.049	0.009	0.959
Styrene			0.334	0.038	−0.040	0.808
Benzene					0.338	0.035

[a] Correlations computed for the 39 control and exposed subjects who had concurrent personal BD and VOC exposure measurements.

on the same day as the benzene, toluene, and styrene measurements. Spearman correlations between these exposures are shown in Table 17 for the 39 subjects with same-day measurements of the four chemicals. Benzene exposure was moderately correlated with BD, styrene, and toluene exposures; and toluene concentrations were unrelated to BD and styrene concentrations. However, the Spearman correlation between BD and styrene was 0.710 ($P < 0.001$); this relation is shown in Figure 9.

Workplace Area Measurements of VOCs

Workplace area measurements were also made for benzene, toluene, and styrene; these mean concentrations were consistent with the measurements made by personal monitoring, although (like the workplace area BD concentrations) they were lower than the personal exposure concentrations (Table 18).

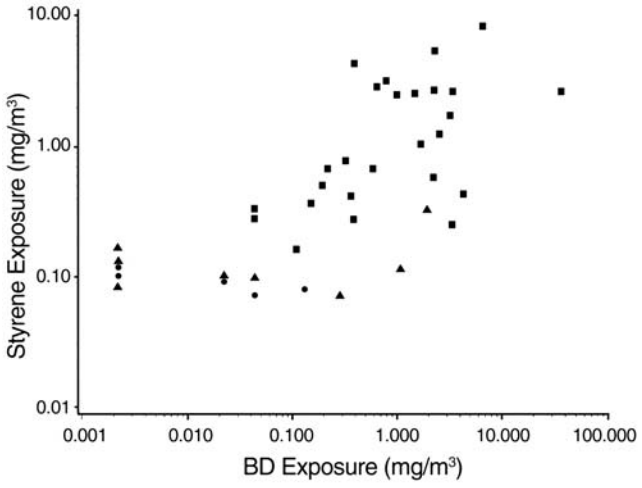

Figure 9. BD and styrene exposure measured on the same day. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

Table 18. Descriptive Statistics for Workplace Area Measurements of VOCs[a]

	Administration	Monomer Unit	Polymer Unit
n Measurements	12	11	14
Benzene			
Mean	0.008	0.016[b]	0.012[b,c]
SD	0.003	0.003	0.005
Minimum	0.004	0.011	0.000
Maximum	0.016	0.021	0.025
50th percentile	0.007	0.016	0.011
90th percentile	0.013	0.020	0.016
Toluene			
Mean	0.032	0.036	0.023[c]
SD	0.041	0.025	0.035
Minimum	0.000	0.014	0.004
Maximum	0.137	0.087	0.118
50th percentile	0.016	0.021	0.010
90th percentile	0.090	0.069	0.093
Styrene			
Mean	0.029	0.038	1.128[b,c]
SD	0.021	0.014	0.681
Minimum	0.001	0.022	0.013
Maximum	0.073	0.061	2.336
50th percentile	0.022	0.035	1.176
90th percentile	0.055	0.060	2.237

[a] All VOCs were measured in mg/m^3.

[b] Significantly different from administration (control) at $P < 0.05$ by Kruskal-Wallis test.

[c] Significantly different from monomer unit at $P < 0.05$ by Kruskal-Wallis test.

DISCUSSION

The average BD exposures of the workers in this study were quite moderate and indicated well-run facilities. The group means were within the range of other studies (Sorsa et al 1994, 1996; Au et al 1995; Tates et al 1996; Ward et al 1996b; Hayes et al 2000), which show that monomer production workers, on average, experience lower exposures than polymerization workers. The control subjects had very low BD exposure levels. The exposure concentrations of styrene, benzene, and toluene were quite low, although styrene levels were significantly higher for the polymerization workers. The correlations between exposures to BD, styrene, and benzene for polymerization workers present difficulties for the evaluation of nonspecific biomarkers because any associations with BD may be due to the effects of styrene. Workplace area measurements of all agents were consistent with the workers' exposures determined from personal monitoring.

The limited availability of sorbent tube samplers necessitated that tubes be reused after they were analyzed and were thus reconditioned at Sheffield. As a consequence, it was not possible to strictly follow the semirandom sampling schedule specified in the exposure assessment protocol. Instead, exposure monitoring was concentrated in intervals at the beginning, middle, and end of the exposure assessment period. Also, because the monitoring of exposed workers was a priority, measurements on control subjects were made during a two-week period midway through the study. Although the gaps in the sampling schedule are of concern, the large variability in exposure levels among different workers on the same day and among different days for the same worker suggests that, on days without measurements, exposures were likely to have been similar to the observed levels. The absence of any apparent time trends in exposure provides further assurance that the scheduling problems did not compromise the validity of the exposure assessment.

The observed variability in exposure among days and among subjects is consistent with the nature of the work performed at the facilities in this study. Much of the time the workers had very little exposure; nearly all of the monomer production and polymerization workers had workshifts during which their exposure levels were comparable to those for administrative control subjects. Higher exposures occurred in conjunction with the tasks a worker performed on particular days and because most tasks are performed independently, exposure levels for different workers on the same day were generally unrelated. BD exposure therefore tends to occur in peaks.

The plan to measure peak levels of BD exposure by small photoionization chambers could not be accomplished primarily because of equipment failure. In an effort to obtain some information about peaks, we asked workers if anything had occurred during their exposure assessment period that caused them to have more exposure to chemicals than usual. Six polymerization workers responded in the affirmative, but only three could remember the date and only one had had a concurrent exposure measurement. His TWA exposure on that day was 0.203 mg/m^3, which was lower than four of his other nine measurements and much lower than his highest measurement of 2.007 mg/m^3. Although a TWA concentration reflects a subject's BD exposure over an entire workshift, days of higher average concentration almost certainly reflect peak exposures because exposure levels in the facilities were usually very low. Unfortunately, without knowing the tasks performed and their duration on those days, it is not possible to estimate

the peak concentrations. The lack of data on peak exposures limited our ability to determine the effects of very high but brief exposures on biomarker production. However, even with more detailed data this would have been very difficult without daily exposure monitoring to ensure that all peaks were observed.

The day-to-day variation in BD exposure demonstrates the importance of obtaining multiple measurements from each worker. In addition to permitting the construction of alternative metrics to reflect different aspects of exposure patterns, multiple measurements would provide a more precise estimate of a worker's average exposure. It is possible, even with 10 measurements per subject during the 60-day assessment period, that some high-exposure days were missed, which would lead to an underestimation of average exposure for some workers. Nevertheless, the current exposure assessment is the most extensive achieved in the BD industry. Most studies of the effects of occupational exposure to BD have obtained only one measurement per person (Sorsa et al 1994, 1996; Au et al 1995; Tates et al 1996; Ward et al 1996b; Hayes et al 2000). A single measurement is likely to be a very poor estimate of a worker's average exposure over time if exposure patterns are similar to those observed in this study.

BIOMARKERS OF EXPOSURE: BD METABOLITES IN URINE

METABOLISM OF BD TO FORM URINARY M2 AND M1

The process of BD metabolism has been described in the earlier Overview section (see Figures 1 and 2). Here we focus on the epoxide metabolite BDO and its hydrolyzed metabolite BD-diol because they are integral to the pathways through which urinary M2 and M1 are formed (Figure 10).

The pathway to form M2 starts with BDO directly conjugating (thus referred to as the "conjugation pathway") with GSH (catalyzed by GST). The GSH conjugate is further metabolized to the corresponding cysteine-*S* conjugate of BDO that is N-acetylated to form the isomeric mixture of M2.

The pathway to form M1 is more complex. It begins with BDO being hydrolyzed (thus referred to as the "hydrolysis pathway") by EH to form BD-diol, which is then conjugated with GSH (catalyzed by GST). The exact mechanism of GSH conjugation of BD-diol is not known. The most common hypothesis is that ADH catalyzes the conversion of BD-diol to 1-hydroxy-2-oxo-3-butene, which would rapidly react with GSH along the unsaturated bond (Sabourin et al 1992; Kemper et al 1996). Once formed, the GSH conjugate is further metabolized to M1, the corresponding mercapturic acid that is excreted in the urine.

Human metabolism of BD has been investigated very superficially. In contrast, the metabolism of BD in mice and rats has been studied in depth. One study exposed mice and rats via a 6-hour inhalation of 200 ppm (442 mg/m^3) [2,3-^{14}C]-BD ([Richardson et al. 1999). Despite the differences in metabolism between rats and mice, all urinary metabolites that could be identified were formed through an epoxy intermediate. These metabolites accounted for more than 98% (in rats) and more than 88% (in mice) of all urinary radioactivity. This makes it likely that, in humans also, epoxides are the major if not only precursor for urinary metabolites. Thus, studying epoxide-derived metabolites as biomarkers of BD exposure in humans is a valid approach.

M2 AND M1 AS BIOMARKERS OF EXPOSURE

Monitoring occupational exposure to BD may be conducted by using one or more urinary metabolites as biomarkers. Mercapturic acids have proved to be very suitable biomarkers for human exposure to a variety of industrial chemicals (eg, allyl chloride, 1,3-dichloropropene, epichlorohydrin, ethylene oxide, benzene) (Van Welie et al 1991;

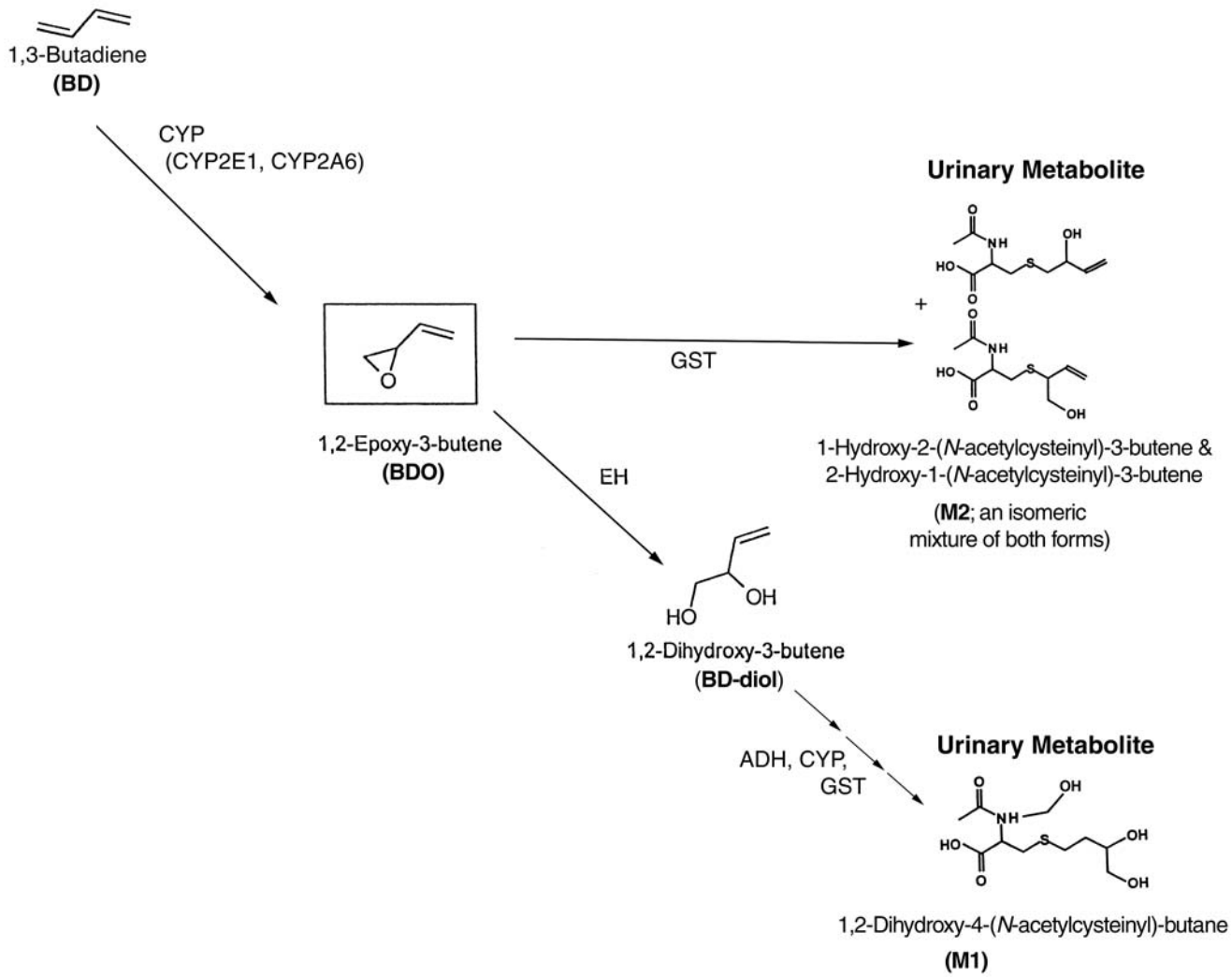

Figure 10. Diagram of only those pathways of BD and BDO metabolism that lead to the formation of the urinary metabolites M2 and M1. This is a portion of Figure 1, which shows more of a simplified metabolic scheme for BD to its various metabolites. The box around a chemical structure indicates that it is an electrophilic (or reactive) epoxide metabolite.

Popp et al 1994; Boogaard and van Sittert 1995, 1996; de Rooij et al 1997a,b). When mercapturic acid metabolites were first proposed as biomarkers for occupational exposure to BD (Osterman-Golkar et al 1991; Osterman-Golkar and Bond 1996), assays were developed to measure M2 and M1 (Bechtold et al 1994). The assay for M1 was designed to be sensitive enough to measure average BD exposures of 3 to 4 ppm (6.6 to 8.8 mg/m^3) during the workshift. However, when it was applied, relatively high concentrations of M1 could also be detected in urine from administrative control subjects. When the assay for M2 was applied, levels of M2 in urine from administrative control subjects were below the LOD of 100 μg/L (Bechtold et al 1994). In the present study, we modified the methods for determining urinary M2 and M1 to increase their sensitivity and then applied them to (1) investigate whether they are sensitive indicators of exposure to BD, and (2) establish the relation between exposure to BD and the selected biomarkers. Characterizing such a relation might permit setting a biological exposure limit (BEL) for BD that would be equivalent to the airborne OEL values.

METHODS

For a review of how urinary samples were collected and handled, see the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Sample Acquisition and Handling / Subjects' Biological Samples.

Synthesis of Reference and Internal Standards

Reference standards of M1 and M2 were prepared as described by Sabourin and colleagues (1992) and Elfarra and associates (1995), respectively. Internal standards were also made: *N*-Acetyl-*S*-(2,3-dihydroxypropyl)-L-cysteine (DHP-MA) was prepared as described by Jones (1975) and used as an internal standard for urinary M1. A deuterium [d_6]-labeled isomeric mixture of M2 ([d_6]-M2) was prepared as described by Elfarra and associates (1995) and used as an internal standard for urinary M2. All reference and internal standards were prepared in methanol in concentrations of 100 μg/L M2 or [d_6]-M2 and 10 mg/L M1 or DHP-MA and stored at −20°C.

Calibration Standards and Curves

An aliquot from each urine sample from all administrative control subjects was pooled and the pool was confirmed to have low natural background levels of M2 and M1; the pool was then spiked with a range of known quantities of the M2 (0–200 μg/L) and M1 (0–20 mg/L) reference standards. These calibration standards were analyzed using gas chromatography with negative-electron-capture ionization and tandem mass spectrometry (GC-NECI-MS/MS) to generate calibration curves; the peak areas of M2 and M1 derivatives increased linearly with M2 and M1 concentrations over the entire range of spiked quantities. The calibration standards were also analyzed with each batch of subjects' urine samples as part of the QC procedures.

QC Urine Standards

From the same pool of administrative control subjects' background urine, two QC urine standards were prepared: one with low concentrations of both M2 and M1 (0.76 ± 0.05 μg/L and 257 ± 27.6 μg/L, respectively) and one with high concentrations of both M2 and M1 (6.75 ± 0.26 μg/L and 1605 ± 90 μg/L, respectively). Both QC urine standards were analyzed 15 times and the mean concentrations of high and low M2 and M1 were calculated to use as assigned QC values. QC urine standards were analyzed at the beginning and end of each day's batch of subjects' urine samples to ensure run-to-run reproducibility.

QC Procedures

Standard operating procedures for handling (see the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Chain of Custody), cleaning up and derivatizing, and analyzing samples were designed and documented in accordance with the requirements of the Shell Research and Technology Centre Amsterdam Management System (NEN-EN-ISO 14001–certified). All staff members were required to keep bound laboratory notebooks acceptable to Good Laboratory Practices (GLP) standards. All data gathered and produced (printed copies and electronic versions) and all notebooks were stored in the Amsterdam central archives when full or not in use. Printed and electronic versions of all finalized data were sent to the central study office in Burlington for statistical analysis and storage.

Before each use, the GC-NECI-MS/MS apparatus was carefully tuned and the sensitivity was checked by measuring the signal-to-noise ratio (S/N) of the *m/z* 272 peak in a selected ion chromatogram after injecting 1 pg octafluoronaphthalene. If the sensitivity was satisfactory (S/N > 10), the system was calibrated and the performance checked by analyzing (1) 100 pg of the two reference and two internal M2 and M1 standards in full-scan mode to verify that selected ion chromatograms of *m/z* 653 (for M1), 639 (for DHP-MA), 441 (for M2), and 447 (for [d_6]-M2) had intensities ranging from 10^5 to 10^6; (2) 100 pg of the two reference and two internal M2 and M1 standards in product ion full-scan mode to verify that product ion spectra contained the parent→daughter ion pairs 653→167 and 653→211 for M1, 639→167 and 639→211 for DHP-MA, 441→175 and 441→176 for M2, and 447→175 and 447→176 for [d_6]-M2; and (3) 10 pg of the two reference and two internal M2 and M1 standards in multiple-reaction monitoring (MRM) mode to verify that S/N for M2 and M1 was ≥ 10.

Additional QC procedures were incorporated before, during, and after analyzing each day's batch of subjects' urine samples. First, at the beginning and end of each series, at least two QC urine standards (both low and high concentrations of M2 and M1) were analyzed. The analytical results of a daily batch were accepted if the deviations in all QC urine standards were less than 5% of their assigned values. Second, a series of calibration standards was randomly spread throughout the samples of the daily batch. About 10% of all subjects' urine samples plus about 10% of all QC urine standards and calibration standards were randomly chosen and divided into duplicates or triplicates. Thus, about 20% of all samples and standards provided QC data. All QC data (from all QC urine standards and from all calibration standards) were checked to ensure run-to-run reproducibility and stability of calibration before data from the daily batch of samples were released for analysis.

Analysis of Urinary M2 and M1

Aliquots (0.1 mL) of internal standards [d_6]-M2 and DHP-MA and 200 mg sodium chloride were added to 1.0-mL

urine samples (pH 2.5); the metabolites were extracted with ethyl acetate containing 20% methanol (v/v). After evaporating the organic phase, metabolites were derivatized by methylation in 1.0 mL methanol:anhydrous HCl (1:10) for 15 minutes at room temperature, followed by pentafluorobenzoylation by reaction with pentafluorobenzoylchloride (7.0 μL) in toluene (400 μL) and pyridine (2.0 μL) for 60 minutes at 60°C. The methylated and pentafluorobenzoylated metabolites were dried and the residue was dissolved in toluene (100 μL).

Derivatized metabolites were analyzed by GC-NECI-MS/MS and MRM on a Finnigan TSQ700 triple-quadrupole mass spectrometer equipped with a Varian 3400 gas chromatograph. The analyses were performed by a 1.0-μL cold on-column injection using a DB-1 column (15 m × 0.32 mm; 0.25 μm film thickness; J&W Scientific) with helium as the carrier gas moving at a flow rate of 1 mL/min. The initial oven temperature was 80°C; after 1 minute, the temperature was increased at a rate of 10°C/min to 320°C and held for 5 minutes. Methane was used as the moderator gas and argon as the collision gas at a source temperature of 120°C and a source pressure of 5000 mTorr (6.67 hPa). Ions (parent→daughters) of the derivatives were monitored at *m/z* 653→167 and 653→211 for M1; *m/z* 639→167 and 639→211 for DHP-MA; *m/z* 441→175 and 441→176 for M2; and *m/z* 447→175 and 447→176 for $[d_6]$-M2.

Concentrations of a metabolite in urine may depend on the rate of urine production; measuring the metabolite in either a too-dilute or too-concentrated urine sample may lead to misinterpretation. Therefore, in all subjects' urine samples, urinary creatinine was also measured using the picrate method on a clinical autoanalyzer (Cobas Mira, Roche). However, when samples in which the creatinine concentrations fell within the range of 4 to 30 mmol/L (the acceptable range) were compared with samples in which the creatinine concentrations fell outside of that range, the results were statistically equivalent. Therefore, we saw no reason to exclude data or samples from the statistical analyses on the basis of creatinine levels.

Statistical Methods

A complete description of all statistical methods is presented in the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Data Processing and Statistical Analysis.

RESULTS

Measurement of Urinary M2 and M1

The LOD for M2 in methanol was 0.1 μg/L; this sensitivity allowed us to quantify the urinary background concentrations. The LOD for M1 in methanol was 5 μg/L; the relatively high urinary background concentrations (mostly > 100 μg/L) could be easily measured.

M2 and M1 urinary metabolites of BD were determined for the 25 administrative control subjects, the 24 monomer production workers, and the 34 polymerization workers. One before-work urine sample from a polymerization worker was not sent to the laboratory due to insufficient volume; therefore, before-work and net (after-work minus before-work) concentrations were determined for 33 of the 34 polymerization workers (see Table 19). The before-work mean concentrations for both metabolites were higher for the polymer group than for monomer or control groups, but group differences were statistically significant only for M2 (Kruskal-Wallis test, $P < 0.001$). As expected, M2 concentrations were 1 to 2 orders of magnitude lower than those observed for M1.

The mean urinary concentration of M1 after work was 353 μg/mL for the control group, 764 μg/L for the monomer group, and 4647 μg/L for the polymer group (Table 19). Despite the variability among values within each group, as reflected by large standard deviations, all group means differed significantly from each other (Kruskal-Wallis test; $P = 0.001$) and paralleled the differences in the mean BD exposures of the groups (see Table 10). The mean urinary concentrations of M2 after work also differed among the exposure groups: 1.70 μg/L for the control group, 9.44 μg/L for the monomer group, and 120.17 μg/L for the polymer group. Again the urinary concentrations were quite variable but group differences were statistically significant (Kruskal-Wallis test; $P = 0.001$) and were in the same rank order as the mean BD exposure concentrations.

Results based on net concentrations (after-work values minus before-work values) were consistent with the after-work results, but the difference in M2 concentrations between monomer and polymer groups was not statistically significant. For the control group, the mean net urinary concentrations for both M2 and M1 were negative and for M1 the value was significantly lower than zero (Wilcoxon signed-rank test, $P = 0.001$). This probably reflects a before-work urinary excretion level somewhat higher than the after-work level for many of the administrative control subjects, as indicated by creatinine concentrations being higher before work than after work (data not shown; Wilcoxon signed-rank test $P = 0.032$). Mean net metabolite concentrations for the monomer and polymer groups were positive; but negative M2 and M1 net concentrations were observed for some individual workers in these groups (the polymerization workers had the most extreme negative values). Large decreases in metabolite concentrations occurred among subjects with high before-work values and

Table 19. Urinary Metabolites by Group[a]

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)[b]
M1			
Before work			
Mean ± SD	571 ± 273	551 ± 290	1493 ± 2081
Median	465	479	610
After work			
Mean ± SD	353 ± 157	764 ± 728[c]	4647 ± 6630[c,d]
Median	314	542	1420
Net (after − before)			
Mean ± SD	−219 ± 274[e]	213 ± 565[c]	2683 ± 5868[c,d]
Median	−162	40	513
M2			
Before work			
Mean ± SD	2.20 ± 1.53	4.16 ± 6.36	25.50 ± 51.03[c,d]
Median	2.00	2.65	5.30
After work			
Mean ± SD	1.70 ± 1.54	9.44 ± 12.97[c]	120.17 ± 228.17[c,d]
Median	1.30	3.70	17.95
Net (after − before)			
Mean ± SD	−0.50 ± 1.22	5.28 ± 8.54[c]	84.26 ± 225.95[c]
Median	−0.70	1.80	10.70

[a] Data are given in µg/L.

[b] One before-work urine sample was not analyzed due to insufficient sample volume; therefore the before-work and net values were calculated for 33 of the 34 polymerization workers.

[c] Significantly different from control group at $P < 0.05$ by Kruskal-Wallis test.

[d] Significantly different from monomer group at $P < 0.05$ by Kruskal-Wallis test.

[e] Significantly lower than 0 at $P = 0.001$ by Wilcoxon signed-rank test.

were not due to differences in creatinine concentrations before and after work (data not shown).

Urinary M2 and M1 metabolite concentrations were normalized to creatinine levels by computing their ratio (micrograms of metabolite per millimole creatinine) for each subject; all statistical analyses were repeated using these values. Results were very similar to those described above, but in general the normalized values had greater variability than the original concentrations. Urinary creatinine concentrations were outside the 4 to 30 mmol/L range in four samples from administrative control subjects, one sample from a monomer production worker, and four samples from polymerization workers. We also performed analyses after excluding those samples because assay results from samples with creatinine concentrations outside the 4 to 30 mmol/L range may be unreliable (Hoet 1996). However, the results were very similar to those obtained using the entire data set.

Correlations Among Urinary Biomarkers and Personal Exposure Monitoring Results

After-work urinary metabolite concentrations were related to BD exposure levels determined on the day of urine collection (Table 20). For the 22 subjects (3 administrative control subjects, 5 monomer production workers, and 14 polymerization workers) for whom same-day exposure data were available, the Spearman correlation (r) with after-work M1 was 0.618 ($P = 0.002$). After-work M2 concentrations showed a very similar relation to same-day exposures ($r = 0.614$; $P = 0.002$). Expanding the analysis to include the additional 22 administrative control subjects for whom same-day BD exposure measurements were not obtained but who presumably had negligible exposures (a total of 44 subjects) did not affect the significance of these relations, although the correlation coefficients were somewhat lower. For the M1 metabolite, the Spearman correlation coefficient was 0.540 for this expanded group

Table 20. Spearman Correlations Between Urinary Metabolites and BD Exposure Measurements

	Same Day Exposure (n = 22)[a]		Same Day Exposure + Control Subjects (n = 44)[b]		Average BD Exposure for All Days (n = 82)[c]	
	r	P	r	P	r	P
M1						
Before work	0.152	0.501	0.179	0.244	0.196	0.077
After work[d]	0.618	0.002	0.540	< 0.001	0.610	< 0.001
Net (after – before)	0.450	0.036	0.367	0.014	0.397	< 0.001
M2						
Before work	0.359	0.101	0.475	0.001	0.542	< 0.001
After work[d]	0.614	0.002	0.483	0.001	0.663	< 0.001
Net (after – before)	0.469	0.028	0.326	0.031	0.469	< 0.001

[a] Includes 3 administrative control subjects, 5 monomer production workers, and 14 polymerization workers whose BD exposure was measured on the same day urine was collected.

[b] Includes the 22 subjects whose BD exposure was measured on the same day urine was collected plus the remaining 22 administrative control subjects.

[c] Includes all subjects except the one polymerization worker described in footnote d.

[d] All sample sizes for after-work urinary metabolites include an additional polymerization worker whose before-work urine was not analyzed due to insufficient sample volume.

($P < 0.001$), whereas for M2 it was 0.483 ($P = 0.001$). The correlations between the logarithms of each after-work urinary M2 or M1 concentration and same-day BD exposures are shown graphically in Figure 11.

Net concentrations of M2 and M1 (after-work values minus before-work values) were also significantly correlated with same-day BD exposure, both when administrative control subjects without these measurements were excluded and when the remaining administrative control subjects were added to the analysis group (Table 20). However, the correlations were all lower than those based on the after-work metabolite concentrations. Before-work urinary metabolite concentrations were not significantly related to same-day BD exposure; however, when administrative control subjects without same-day BD exposure were included in the analysis, the urinary M2 before-work concentration was significantly correlated with exposure ($r = 0.475$, $P = 0.001$).

Table 20 also includes correlations between urinary metabolite concentrations and subjects' average BD exposures for the entire assessment period. After-work and net M2 and M1 metabolite concentrations correlated as well with average exposures as they did with same-day exposures. Average exposure was also significantly correlated with before-work M2 concentration ($r = 0.542$, $P < 0.001$), but not with before-work M1 concentration.

Similar correlation analyses were done using urinary metabolite concentrations expressed as micrograms of metabolite per millimole creatinine, but correlations with same day exposure were always lower.

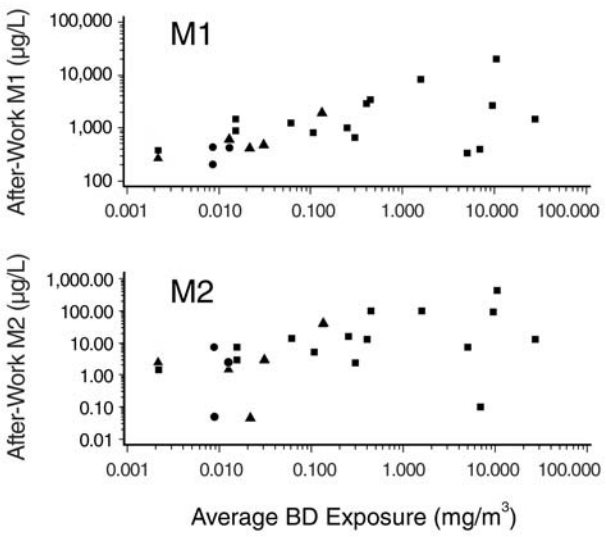

Figure 11. Urinary M1 or M2 metabolite concentrations vs same-day BD exposure. Comparisons for 3 administrative control subjects, 5 monomer production workers, and 15 polymerization workers whose BD exposure was measured on the same day urine was collected. Note that the scales on the y axes differ between the top and bottom panels. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

Influence of Genotypes and Metabolic Pathways (GSH Conjugation vs EH Hydrolysis)

Statistical analyses were performed to examine the possible influence of *GSTM1* and *GSTT1* genotypes on urinary excretion concentrations of the M2 and M1 metabolites. No significant effects of genotype expression were detected in any of the groups or through the entire study population as a whole. We also found no significant effect of smoking on either the M2 or M1 metabolite concentration, and no significant interactions between exposure group and either genotype or smoking.

M2 represents the conjugation pathway of BD metabolism because it results from the detoxification of BDO by direct GSH conjugation, whereas M1 represents the hydrolysis pathway of BD metabolism because it results from detoxification of BDO by epoxide hydrolysis as the first step. Hence, the ratio of urinary M2/(M1 + M2) is directly related to the activity of the GSH conjugation pathway mediated by GST and inversely related to the activity of the hydrolysis pathway mediated by EH. The ratio M2/(M1 + M2) was determined for the BD urinary metabolite data to investigate whether this ratio is influenced by BD exposure. When M2 and M1 concentrations were determined on after-work urine samples, the mean ± SD ratio was 0.0049 ± 0.0043 for the control group, 0.0115 ± 0.0110 for the monomer group, and 0.0182 ± 0.0153 for the polymer group. The rank order of these mean ratios, polymer group > monomer group > control group, is the same as the rank order of group mean BD exposures. The control group mean differs significantly from both the monomer and the polymer group means (Kruskal-Wallis test; $P < 0.001$). The after-work ratio M2/(M1 + M2) was significantly correlated with average BD exposure over the entire assessment period ($r = 0.539$, $P < 0.001$) and with same-day exposure concentration among workers who had this measurement on the first day of urine sampling ($r = 0.451$, $P = 0.031$).

In Table 21, mean values for the ratio M2/(M1 + M2) are given for *GSTM1* and *GSTT1* genotypes. The mean ratio for workers of the *GSTM1*-null genotype was always lower than the mean ratio for workers in the same group who had the positive phenotype; when all subjects were included, the difference between the null and positive *GSTM1* genotypes was statistically significant (ANOVA, $P = 0.036$). We found no significant exposure-group–by–genotype interaction, which indicates that the size of the difference between the null genotype and positive genotype did not vary significantly among the three exposure groups. However, regression analysis of M2/(M1 + M2) ratios on BD exposure by genotype (assuming a common intercept and excluding unexposed administrative control subjects) showed a significantly positive difference between slopes for after-work values for workers of *GSTM1*-positive genotype ($P = 0.027$) (Figure 12). This difference in slope was not significant, however, when the logarithms of the ratios were used in the analysis. (We had compared *GST* genotypes in unexposed administrative control subjects to determine that it is appropriate to assume a common intercept at low BD exposure levels for the regression analysis of these genotypes.)

Workers with the *GSTT1*-null genotype also had lower mean ratios than workers in the same group with the *GSTT1*-positive phenotype, but the average difference across groups was not statistically significant and we

Table 21. Urinary Metabolite Ratio M2/(M1 + M2) After Work by Group and Genotype[a]

	Control ($n = 25$)	Monomer ($n = 24$)	Polymer ($n = 34$)	All Groups ($n = 83$)
GSTM1				
n	16	14	23	53
Positive	0.0052 ± 0.0041	0.0137 ± 0.0131	0.0211 ± 0.0168	0.0143 ± 0.0146
n	9	10	11	30
Null	0.0044 ± 0.0048	0.0085 ± 0.0067	0.0121 ± 0.0097	0.0086 ± 0.0079[b]
GSTT1				
n	20	20	30	70
Positive	0.0054 ± 0.0046	0.0130 ± 0.0116	0.0190 ± 0.0158	0.0134 ± 0.0134
n	5	4	4	13
Null	0.0029 ± 0.0016	0.0044 ± 0.0013	0.0117 ± 0.0096	0.0060 ± 0.0063

[a] Data are presented as the mean ± SD for the number of subjects in the row above.

[b] Significantly different from positive genotype at $P = 0.036$ by two-way ANOVA. No significant exposure-group–by–genotype interactions were found.

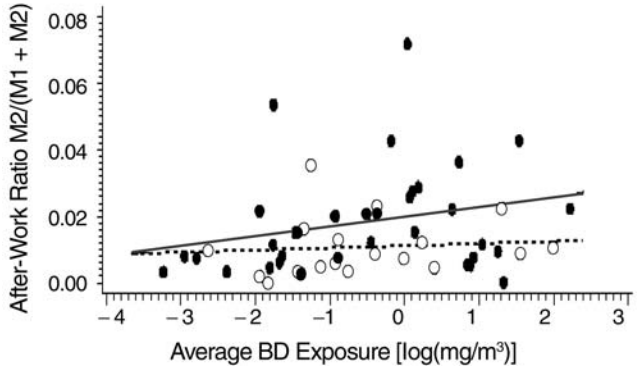

Figure 12. Urinary metabolite ratio M2/(M1 + M2) vs average BD exposure by *GSTM1* genotype. ● and solid line indicate *GSTM1*-positive phenotype; ○ and broken line indicate *GSTM1*-null genotype. Slopes differ significantly ($P = 0.027$) as determined by regression analysis assuming a common intercept for both genotypes.

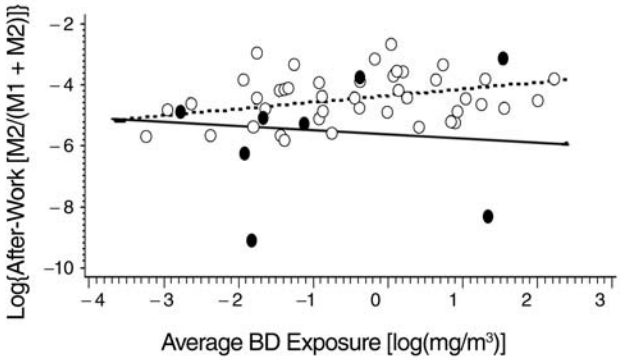

Figure 14. Urinary M2/(M1 + M2) vs average BD exposure by *CYP2E1* genotype. ● and solid line indicate *C/D* genotype; ○ and broken line indicate *D/D* genotype. Slopes differ significantly ($P = 0.013$) as determined by regression analysis assuming a common intercept for both genotypes.

found no significant exposure-group–by–genotype interaction. We also noted no significant slope differences in the regression analysis using this genotype.

Workers were also genotyped for *CYP2E1*, *EH*, and *ADH* polymorphisms. *EH* and *ADH* genotypes showed no association with M1 or M2 concentrations or the ratio. However, regression analysis on BD exposure by genotype revealed a significantly positive slope difference for both after-work M2 concentrations ($P = 0.030$; Figure 13) and M2/(M1 + M2) ratios ($P = 0.013$; Figure 14) for workers who are homozygous for the *CYP2E1 D/D* intron 6 polymorphism compared with workers who are heterozygous for *C/D*. The slope difference for the ratio, however, was only significant when the logarithm of the ratio was used in the analysis. Although the functional significance of the *D* mutation is not certain in the homozygous form, it was associated with greater in vivo production of the M2 metabolite of BD in these workers.

We found no significant differences in the M2/(M1 + M2) ratios for current smokers compared with nonsmokers in this study population.

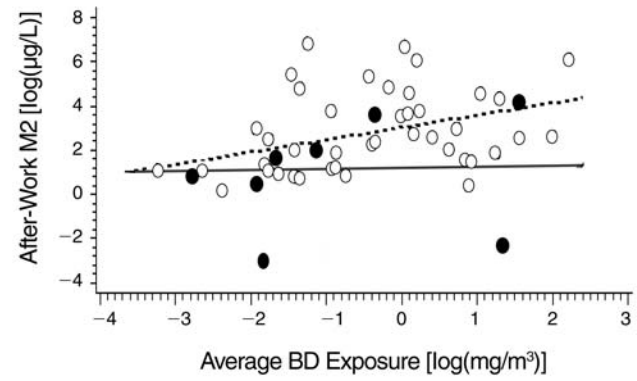

Figure 13. Urinary M2 vs average BD exposure by *CYP2E1* genotype. ● and solid line indicate *C/D* genotype; ○ and broken line indicate *D/D* genotype. Slopes differ significantly ($P = 0.030$) as determined by regression analysis assuming a common intercept for both genotypes.

DISCUSSION

Because of the potential carcinogenicity of BD to humans, it is important to minimize occupational and environmental exposures. To evaluate the effectiveness of control measures, reliable biomarkers are needed that allow detection of low-level BD exposure in occupationally exposed workers. In addition, measuring biomarkers may provide insight into the human metabolism of BD and allow a comparison of metabolism among species (mouse, rat, human), which would be useful in assessing cancer risk in humans. We modified the urinary M2 and M1 tests, which were first used by Bechtold and coworkers (1994) as biomarkers of exposure to BD, to enable detection of the low BD exposure levels now present in occupational settings. In the method as developed in Amsterdam for determining urinary M2, the sensitivity was improved enough to allow detection of low natural background levels. In the unexposed administrative control subjects, concentrations ranging from < 0.1 to 8.2 µg/L were measured. Natural background levels of M1 were markedly higher than those of M2 (generally > 100 µg/L). From the background value, the 97.5 percentile concentration of M2 was 5.5 µg/L and of M1 was 700 µg/L. This means that workers whose after-work urine samples contained M2 or M1 in concentrations that exceeded these values were most likely exposed to BD.

Results for both urinary biomarkers correlated well with the personal exposure monitoring results for BD. The best correlations were obtained using the concentrations of M2 or M1 measured in after-work samples. Surprisingly, the correlation between personal BD exposure and the net increase of M2 or M1 was not as good as that obtained with after-work samples, even when only urinary samples and exposure data that were collected on the same day were used in the analysis. In urine samples from polymerization workers collected before work on the first urine sampling day, M2 and M1 concentrations were both higher than in samples from administrative control subjects. However, only for M2 was this higher value statistically significant, which indicates that M2 from previous BD exposure had not yet been completely eliminated after the presumed 48-hour period without occupational BD exposure.

When we designed this study, we assumed that the half-lives of M2 and M1 would be short enough to allow a complete washout in a period of 2 days. The elimination half-lives for the mercapturates of several industrial chemicals have been determined through biological monitoring of operators and found to be short. For compounds that do not require a first phase of metabolism but undergo direct GSH conjugation (such as allyl chloride, 1,3-dichloropropene, and epichlorohydrin), half-lives range from 2.5 to 5.0 hours (Van Welie et al 1991; de Rooij et al 1997a,b); this indicates that the excretion of mercapturates from humans is very rapid. For compounds such as BD that require oxidation metabolism before GSH conjugation and subsequent second-phase metabolism to mercapturates, somewhat longer half-lives were observed, but still short enough to allow a washout in 2 days. For benzene, for instance, a half-life of 9.1 hours was determined (Boogaard and van Sittert 1995, 1996). Thus far, the only exception reported is dimethylformamide, which is metabolized via *N*-methylformamide and methyl isocyanate to *N*-methylcarbamoyl mercapturic acid, and then excreted in the urine with an apparent half-life of 23 hours. In this case the long half-life can be explained by the rate-limiting reversible protein binding of the methylisocyanate intermediate before the GSH conjugation (Mraz et al 1991; Mraz and Nohova 1992); but such a protein binding is not expected for BD.

As previously noted, urine samples were collected from workers on the last 3 days of exposure assessment and the day-2 and day-3 samples were stored for possible analysis later. In an attempt to further characterize the unexpectedly prolonged half-lives of the M2 and M1 metabolites, attempts were made to analyze day-3 samples in a different laboratory, but this was unsuccessful.

The measurement of relatively high natural background levels of M1 in urine from individuals with no known exposure to BD demonstrates that M1 is not only produced from exposure to BD but also from other sources. After BD exposure, M1 is formed by hydrolysis of BDO to BD-diol and then conjugation of BD-diol with GSH. It is unknown where the background levels of M1 originate. We postulated that endogenous M1 originates from endogenous BD-diol. What these natural background levels imply for cancer risk from BD exposure are currently unknown.

The data on biomarkers obtained in this study can be used to explore relative pathways of metabolism of BD in humans, which can be compared with metabolism data from rats and mice. First, that the M1 values were so much higher than the M2 values (usually one to two orders of magnitude higher in the same urine sample) indicates that hydrolysis is the primary route of detoxification in humans. Second, the linear regression models of the relations between same-day BD air exposure and urinary M2 or M1 indicate that the proportion of inhaled BD that is excreted as M2 or M1 in urine decreases with increasing BD exposures, probably because other metabolic pathways take precedence. Third, because M2 is formed through direct GSH conjugation and M1 through EH-catalyzed hydrolysis and then GSH conjugation, the ratio M2/(M1 + M2) is a direct measure for the importance of the GSH conjugation pathway in the detoxification of BDO. Table 22 summarizes the excretion of urinary metabolites from rat, mouse, monkey, and human after exposure to BD. The ratio M2/(M1 + M2) ranks human < monkey < rat < mouse for the conjugation pathway, which is consistent with previous reports (Sabourin et al 1992; Bechtold et al 1994; Dahl and Henderson 2000). This shows that hydrolysis is a much more important pathway in primates than in rodents.

The ratio M2/(M1 + M2) was lower in all three groups of workers with null genotypes of both *GSTM1* and *GSTT1*. This indicates that, most likely, workers with both *GST* genotypes are catalyzing the direct conjugation of BDO with GSH. Although for workers with either the *GSTM1*-null or *GSTT1*-null genotype the mean ratio was always lower than for the workers with the positive genotype, the effect was only statistically significant for workers with the *GSTM1* genotypes. The effects were not very strong, again indicative of the relatively minor importance of the conjugation pathway compared with the hydrolysis pathway for BDO.

Other researchers have hypothesized that the formation of M1 involves ADH (Sabourin et al 1992; Kemper et al 1996). However, in this study, neither the excretions of M2 and M1 nor the ratio M2/(M1 + M2) showed any correlation with *ADH* genotypes. Although no effect of *ADH* genotypes on the ratio M2/(M1 + M2) was observed, we cannot rule out that ADH is involved in the formation of

Table 22. Comparison of Urinary Metabolite Excretion and Metabolite Ratios Among Human, Monkey, Rat, and Mouse After BD Exposure

	M2 (Conjugation Pathway from BDO)	M1 (Hydrolysis Pathway from BDO)	M2/(M1 + M2)
Human			
Present study[a]			
BD for 8 hours at 2.21 mg/m^3 (1 ppm)			
Amount of metabolite (µg/L)	21.5	2257	0.0094
Monkey			
Sabourin et al 1992			
BD for 2 hours at 663 mg/m^3 (300 ppm)			
Percentage of total metabolites	6	55	0.098
Rat and Mouse			
Bechtold et al 1994			
BD for 4 hours at 25.86 mg/m^3 (11.7 ppm)			
Rat: Amount of metabolite (nmol)	150	156	0.49
Mouse: Amount of metabolite (nmol)	34	10.3	0.767
Rat and Mouse			
Richardson et al 1999			
[^{14}C]BD for 6 hours at 442 mg/m^3 (200 ppm)			
Rat: Percentage of total metabolites	18.6	17.5	0.515
Mouse: Percentage of total metabolites	16.6	10.1	0.622

[a] The monkey, rat, and mouse studies reported here each used a predetermined and fixed BD exposure level. In the current study, each human subject was exposed to a different concentration of BD. Therefore, to more easily compare the human data with those from other species, we chose a concentration of 2.21 mg/m^3 (1 ppm) because, first, it is commonly used in similar calculations for carcinogens such as ethylene oxide and propylene oxide; and second, it is the internal exposure limit for BD used by Shell, where this work was done. Furthermore, we arrived at the M2 and M1 values by substituting the value of 2.21 in the equations representing the regressions depicted in Figure 11. (These equations ignore incomplete washout, which potentially affects the data points from which the equations were derived.)

M1. For *ADH2*, which was one of the *ADH* polymorphisms we studied, very few workers carried the variant allele (see the earlier section Workers' Exposure Groups, Personal Characteristics, and Metabolic Genotypes / Metabolic Genotypes / Results: Genotypes).

After exposure to [2,3-^{14}C]BD for 6 hours, the ratio of [total metabolites formed via GSH pathway]/[total metabolites formed via hydrolytic plus GSH pathways] was even smaller in rats and greater in mice than the ratio M2/(M1 + M2) (Richardson et al 1998c, 1999; van Sittert et al 2000). These findings, along with those from the current study and from Sabourin and associates (1992), also indicate that, in primates compared with rodents, a much greater proportion of BDO is metabolized by hydrolysis and a much smaller proportion is detoxified via direct conjugation with GSH. Also in the current study, however, the M2/(M1 + M2) ratio for all subjects was found to increase at increasing BD exposures, even though the changes were small. Regardless, these data indicate that, as the BD exposure concentrations increased, a somewhat smaller proportion of BDO was hydrolyzed into BD-diol and a somewhat greater proportion was detoxified by direct conjugation with GSH.

An unexpected observation was an association between the *CYP2E1 D/D* genotype and increased production of the M2 metabolite with higher M2/(M1 + M2) ratios relative to BD exposure levels. The significance of this remains to be defined. Comparative studies in transgenic rodents may help to clarify this and further elucidate the metabolic pathways of BD. This finding illustrates how molecular epidemiologic findings in humans can be used, along with animal data, for the overall toxicologic evaluation of a chemical.

From the findings described above about the metabolism of BDO in humans, rats, and mice after exposure to BD, it may be expected that the proportion of BDO that undergoes further oxidation to BDO_2 is of the rank order of mouse > rat > human. Even if BDO_2 is formed in humans, one would expect it to be rapidly hydrolyzed to BDO-diol, or erythritol, or both, or to be further metabolized by direct conjugation with GSH (Boogaard and Bond 1996;

Boogaard et al 1996) and released as a detoxification product. Thus, the extent to which BDO_2 would be available, if at all, to react with either DNA or Hb is very small. This is, indeed, in line with the results on Hb adducts of metabolites of BD that were obtained in this study (see the next section Biomarkers of Exposure: Hb Adducts), which also led to the conclusion that BDO-diol and not BDO_2 is the most important circulating reactive metabolite of BD.

BIOMARKERS OF EXPOSURE: Hb ADDUCTS

Exposure assessment is one of the most important but least accurate parameters in epidemiologic studies. Exposure is often estimated only from job descriptions; and industrial hygiene measurements are usually made in designated areas of the workplace and may not reflect individual worker's exposures. In addition, peak exposures may not be captured in routine monitoring.

Measurements of Hb adducts offer some important advantages over industrial hygiene measurements and other measurements of internal dose:

- Blood samples are readily available and contain large amounts of Hb.
- The analysis of protein adducts is highly sensitive and specific. Small Hb adducts are not repaired and therefore accumulate for the life span of the erythrocyte (120 days in humans); this allows studies to be carried out at low exposure levels.
- Studies of Hb adducts in humans, which seek to integrate exposure over time, can provide considerably improved exposure assessments because the adducts accumulate for about 120 days.
- Hb adducts provide a measure of the dose of reactive chemicals in laboratory animals and in humans, thereby eliminating some of the difficulties in extrapolating from high doses to low doses and across species (Osterman-Golkar et al 1993).
- Hb adducts provide information on the internal dose of the reactive intermediates of a chemical; in the case of BD, these intermediates have vastly different potencies as genotoxins.

Nevertheless, Hb adducts also have clear limitations. They provide information on the average exposure of an individual over the life span of the erythrocyte, but they do not provide information on tissue-specific alkylation. For example, inhalation exposure to propylene oxide yields one value for its Hb adduct hydroxypropylvaline; whereas the same exposure produces 43-fold differences in DNA adducts in different tissues (Ríos-Blanco et al 2000). Hb adducts do not directly reflect persistent DNA damage. As mentioned above, Hb adducts are not repaired, whereas DNA adducts are subject to repair; unless exposure occurs under very similar patterns each day, the relations between Hb and DNA adducts become extremely complex. Thus, Hb adducts provide excellent measures of systemic exposure, but have clear limitations in predicting the extent of DNA damage associated with that exposure.

The BD metabolite BDO (see Figure 15) has been shown

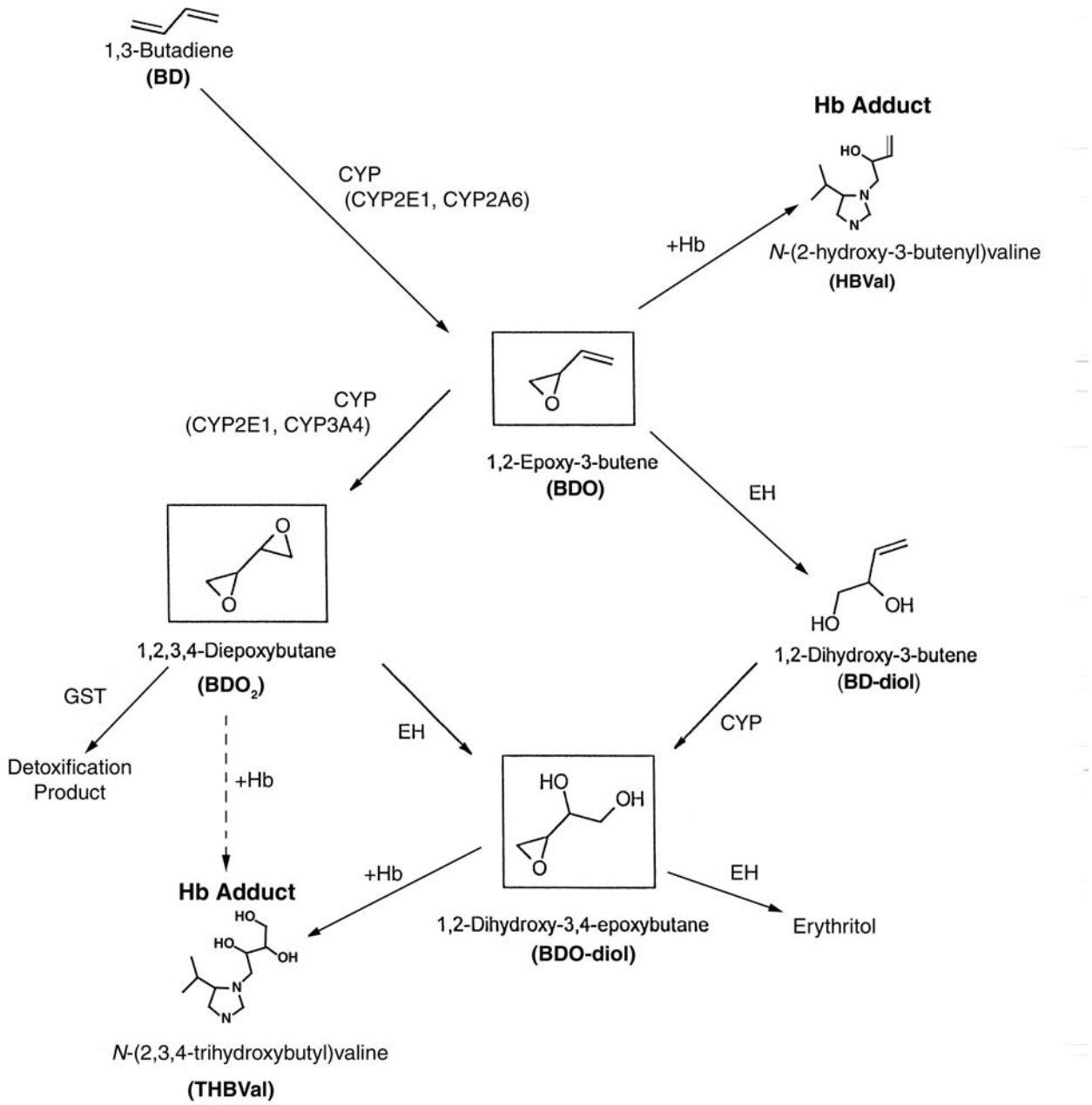

Figure 15. Diagram highlighting the metabolism of BD to BDO, BDO_2, and BDO-diol, which lead to the formation of the Hb adducts HBVal and THBVal. This is a portion of Figure 1, which shows more of a simplified metabolic scheme for BD to its various metabolites. The box around a chemical structure indicates that it is an electrophilic (or reactive) epoxide metabolite; arrow with broken lines indicate some uncertainty about this pathway.

to react with Hb to form HBVal adducts. Two major and two minor peaks have been identified using GC-MS. The two major peaks were shown to be diastereomers resulting from attack on the *N*-terminal valine-NH_2 at the C_1 position of BDO (Osterman-Golkar et al 1991, 1993; van Sittert and Van Vliet 1994). Adduct concentrations of 1 to 3 pmol/g globin have been recorded in humans (nonsmokers) working in a production area with ~1 ppm (2.21 mg/m^3) BD exposure levels. Adducts have also been measured in cigarette smokers not occupationally exposed to BD. The reported adduct levels were lower in humans than in mice and rats exposed to 2 ppm (4.42 mg/m^3) BD, and were

much lower than hydroxyethylvaline adducts associated with occupational exposures to ethylene oxide and ethylene (Osterman-Golkar et al 1991, 1993; van Sittert and Van Vliet 1994). Albrecht and colleagues (1993) reported adduct amounts five times higher in mice than in rats (17 and 3.5 nmol/g globin, respectively, at exposure levels of 500 ppm [1105 mg/m^3], 6 hours/day, for 5 days), although the diastereomers were not resolved by GC-MS.

It is clear that BD exposure results in a nonlinear dose-response that is characteristic of metabolic activation reaching saturation, and that mice have higher amounts of the HBVal adducts than rats (Albrecht et al 1993; La and Swenberg 1997; Koc et al 1999). This conclusion is also supported by two studies on the induction of micronuclei in rats and mice in that micronuclei could not be induced in rats (Adler et al 1994; Autio et al 1994). In pilot studies, we compared male and female rats and mice exposed to 1000 ppm (2210 mg/m^3) BD for 13 weeks and found that females had higher levels of HBVal adducts than males (Tretyakova et al 1996). This was confirmed in a larger study (Swenberg et al 2000a) and in subsequent comparisons of rats and mice (Upton et al 2000). All of the Hb studies have utilized the modified Edman degradation method of Törnqvist and associates (1986), which is based on GC-MS measurements that use an internal standard of [d_4]-*N*-(2-hydroxyethyl)valine, [^{14}C]-*N*-(2-hydroxypropyl)valine, or *N*-(2-hydroxy-3-butenyl)valine-glycine-glycine, or an external standard of *N*-(2-hydroxy-3-butenyl)-[$^{13}C_5$]-valine.

A second Hb adduct of BD, THBVal, was initially thought to arise from BDO_2, which was subsequently hydrolyzed to the adduct. Two facts about BDO_2 highlighted the importance of developing methods to quantify the relative amounts of Hb adducts so that comparisons could be made among species: BDO_2 is formed in greater amounts by the mouse than by the rat; and the mutagenicity of BDO_2 is much greater than other BD metabolites. Pérez and coworkers (1997) reported the formation of THBVal adducts of Hb in rats and two humans and provided evidence that they were primarily formed by BDO-diol rather than by BDO_2. Furthermore, the THBVal adducts were formed in greater amounts than previous measurements of HBVal adducts. The authors concluded that BDO-diol appeared to be an important metabolite of BD. In rodents exposed to [4-^{14}C]BDO (1–50 mg/kg by intraperitoneal injection), both HBVal and THBVal increased in nonlinear fashion with dose. In both rats and mice, the concentration of THBVal was two orders of magnitude higher than the concentration of HBVal. In rats, the ratio THBVal:HBVal was constant with increasing dose; in mice this ratio was about twice as large and decreased with increasing dose, which suggests metabolic saturation (Richardson et al 1996, 1997, 1998b). In rats and mice exposed by nose-only inhalation to 200 ppm (442 mg/m^3) [2,3-^{14}C]BD for 6 hours, the ratio of THBVal:HBVal was higher in mice than in rats (Richardson et al 1998a,b; van Sittert et al 2000).

The THBVal adducts were further explored in human and animal studies by Swenberg and colleagues (2000a) and in a molecular epidemiology study by Hayes and associates (2000). Both adducts appeared to be excellent biomarkers of exposure to BD, but data have not been analyzed to systematically demonstrate the relations between HBVal and THBVal in humans. The present investigation was the first study to show that the ratio THBVal:HBVal is approximately 400 in humans exposed to $<$ 2.21 mg/m^3 ($<$ 1 ppm) BD.

Furthermore, the presence of THBVal adducts has been clearly demonstrated in unexposed animals and humans (Richardson et al 1998a,b; Hayes et al 2000; Swenberg et al 2000a). The sources of the endogenous THBVal are not presently known, but it may not actually arise from exposure to BD.

Currently no methods are available to quantify specific Hb adducts of BDO_2. A cyclic adduct on the *N*-terminus of valine [*N,N*-(2,3-dihydroxybuta-1,4-diyl)valine] has been proposed by Rydberg and coworkers (1996). A major problem in developing ultrasensitive methods to identify this adduct is that the modified Edman degradation cannot be applied. A method involving acid hydrolysis and derivatization for gas chromatography with electron-capture negative chemical ionization and high-resolution mass spectrometry (GC-ECNCI-HRMS) is being developed. When human globin was exposed to BDO_2 (~0.02 mmol), elevated levels of *N,N*-(2,3-dihydroxybuta-1,4-diyl)valine (6 nmol/mg globin) were demonstrated using GC-MS/MS (Ranasinghe et al 2000).

Several investigators have shown that mice produce much greater amounts of BDO_2 than rats. Metabolism studies of BD using human liver suggest that humans are more like rats than mice in the amount and type of metabolites formed; however, considerable interindividual variation has been noted (Seaton et al 1995; Boogaard and Bond 1996; Boogaard et al 1996).

Recent molecular dosimetry studies on DNA adducts of BD in rodents have shown that BDO-diol is the major electrophilic metabolite of BD and accounts for ~98% of the adducts formed (Koc et al 1999; Koivisto et al 1999; Van Sittert et al 2000; Swenberg et al 2000b; Boogaard et al 2000, 2001, unpublished results 2003). This was a highly significant finding; none of the metabolism studies had measured BDO-diol and it is the weakest mutagen of the three electrophilic metabolites, being 1/200th as potent as BDO_2 (Cochrane and Skopek 1994a,b). Although it can be formed from either oxidation of BD-diol or hydrolysis of BDO_2, the molecular dosimetry studies of DNA adducts

strongly support the hydrolysis of BDO_2 as the primary pathway (Koc et al 1999; Boogaard et al unpublished results 2003). The previous measurements of BDO and BDO_2 are thought to represent the amounts of these metabolites that escape from the endoplasmic reticulum before hydrolysis of BDO_2 to BDO-diol. The species differences for BDO_2, with mice having much higher amounts, represents the greater role of GSH detoxification in mice compared with rats and humans, in which EH is the dominant pathway for detoxification of BD.

Hb adducts may be considered to be surrogates for the functionally important DNA adducts. Although not investigated in the current study population, the identification and quantification of DNA adducts formed by BD and its metabolites have been actively studied by several research teams. DNA adducts have been characterized at the N7 position of guanine and the N1, N3, and N6 positions of adenine (Citti et al 1984; Leuratti et al 1994; Neagu et al 1995; Tretyakova et al 1996, 1997a,b,c, 1998; Koivisto et al 1996, 1998b; Boogaard et al 1998, 2000, 2001, unpublished results 2003). Multiple DNA adducts are formed at each of the above positions because attack at the C_1 and C_2 positions of BDO, BDO-diol, and BDO_2 are possible. This is further complicated by diastereomers if nucleosides or nucleotides are being measured, and by stereoisomers of the trihydroxybutyl-adenine and trihydroxybutyl-guanine adducts formed by BDO-diol and isomeric or *meso*-BDO_2.

The molecular dosimetry study of Koc and coworkers (1999) compared BD-DNA adducts in rats and mice across exposures ranging from 20 to 625 ppm (44.2–1381.3 mg/m^3) for 4 weeks. Using liquid chromatography with tandem mass spectrometry (LC-MS/MS), the study demonstrated that the trihydroxybutyl adducts at N7 of guanine (THB-G) were formed in much greater amounts than the hydroxybutenyl adducts at N7 of guanine {HB-G; the total of N7-(1-[hydroxymethyl]-2-propenyl)guanine and N7-(2-hydroxy-3-butenyl)guanine} in both rats and mice. In addition, the study demonstrated that the exposure-response curve for THB-G was nonlinear, a result of metabolic activation reaching saturation. In contrast, the exposure-response curve for HB-G was linear. This study also strongly suggested that BDO-diol was the main electrophile to form THB-G. It also showed that similar numbers of adducts were present in all tissues examined, which suggests that the electrophilic metabolites circulate in the blood.

Koivisto and colleagues (1999) confirmed these findings using ^{32}P-postlabeling. When rats and mice were exposed via nose only to [2,3-^{14}C]-BD (200 ppm [442 mg/m^3] for 6 hours), the major DNA adduct formed in liver, lung, and testes was also THB-G. The concentrations of THB-G were more than an order of magnitude higher than concentrations of HB-G in both mice and rats. In rats and mice exposed to ^{14}C-BDO, HB-G was formed; the levels in rats were 2 to 3 times higher than those in mice. THB-G was also formed, but at much higher concentrations (Boogaard et al 1998, 2000, 2001, unpublished results 2003). In vitro reactions of 2′-deoxyguanosine and DNA with the stereoisomers of BDO-diol, isomeric BDO_2, and *meso*-BDO_2 showed specific formation of the THB-G adducts from the different epoxides; this confirms that BDO-diol is the most important epoxide of BD with regard to macromolecular binding (Boogaard et al 2000, 2001, unpublished results 2003).

In the present study, we modified the methods for determining HBVal and THBVal to gain sensitivity and then applied them to:

1. investigate the sensitivity of the Hb adducts as biomarkers of human exposure to BD;
2. establish the relation between workplace exposure to BD and the levels of HBVal and THBVal (determining such a relation may permit setting a BEL for BD that corresponds with airborne OEL values);
3. investigate the correlation between HBVal and THBVal levels;
4. use the molecular dosimetry of Hb adducts as an individual's internal dosimeter of BD exposure to investigate exposure-response relations for genotoxicity studies in the same individual; and
5. examine the 95% CI for THBVal as a predictor of interindividual differences in the metabolism of BD.

MATERIALS AND METHODS FOR HBVal ANALYSIS

Synthesis of Reference and Internal Standards

HBVal in globin was used as the reference standard and prepared as described by Osterman-Golkar and Bond (1996).

An isomeric mixture of [d_6]HBVal in globin was used as an internal standard and prepared as follows: [d_6]-Labeled BDO was prepared from [d_6]BD as described by Handley (1994). A 70-μL aliquot of a solution of [d_6]BDO in DMSO (30 mg BDO in 300 μL DMSO) was added to washed human erythrocytes and then placed in a shaker incubator at 35°C for 16 hours. Globin was extracted from the erythrocytes and purified as described previously (Mowrer et al 1986). The level of [d_6]HBVal in globin was determined using [^{14}C]-labeled HBVal in globin as a reference standard in the Edman degradation method. The amount of [^{14}C]HBVal in globin was measured by protein hydrolysis and chromatography. Using this procedure, the level of [d_6]HBVal in globin was determined to be 951 nmol/g globin.

Reference and internal standard solutions were prepared in formamide and stored at −20°C.

Calibration Standards and Curves

Calibration curves with known quantities of HBVal (0–50 pmol/g globin) were prepared from the calibration standard of HBVal (200 pmol/g globin/mL formamide). The peak areas of the pentafluorophenylthiohydantoin (PFPTH) derivative of HBVal increased linearly with HBVal in globin concentrations over the entire range.

QC Procedures

We followed the QC procedures outlined for the urinary metabolites M2 and M1 (see the earlier section Biomarkers of Exposure: BD Metabolites in Urine / Methods / QC Procedures / second paragraph) with the following exception. Once the GC-MS/MS apparatus was tuned and the sensitivity was satisfactory, the system was calibrated and the performance checked by analyzing (1) 70 pg of each of the reference and internal standards in full-scan mode to verify that selected ion chromatograms of *m/z* 374 and 380 had intensities ranging from 10^5 to 10^6; (2) 70 pg of each of the reference and internal standards in product ion full-scan mode to verify that product ion spectra contained the parent → daughter ion pairs 380 → 320 and 374 → 318; and (3) 0.7 pg of each of the reference and internal standards in MRM mode to verify that S/N ratios for both HBVal and [d_6]HBVal were ≥ 5.

Each day at least two QC standards, a high and a low concentration standard, were analyzed following the procedures outlined for urinary metabolites in the earlier section Biomarkers of Exposure: BD Metabolites in Urine / Methods / Calibration Standards and Curves; and QC Procedures / third paragraph.

HBVal Analysis

HBVal was measured as described previously (Richardson et al 1996, 1998a) except that [d_6]HBVal was used as an internal standard and that analysis of the samples was carried out by gas chromatography with negative chemical ionization and tandem mass spectrometry (GC-NCI-MS/MS) with MRM. In brief, globin was isolated and the internal standard solution (10 pmol [d_6]HBVal/g globin) was added to 200-mg globin samples dissolved in formamide (6 mL). The pH was adjusted to between pH 6.6 and 7 and pentafluorophenylisothiocyanate (PFPITC; 30 μL; Fluka, Buchs, Switzerland) was added and the mixture incubated (about 16 hours) in the dark at room temperature. The reaction mixture was heated to 45°C for 90 minutes and then cooled to room temperature; 1.0 mL water was added to the PFPTH derivatives, followed by 2.0 mL ether. After shaking and centrifugation for 10 minutes at 1000*g*, the ethereal phase was removed; further aliquots of water (1.0 mL) and ether (3.0 mL) were added and the partition was repeated; the ethereal phases were combined and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 1 to 2 mL toluene and the solution partitioned with 2.0 mL water; aqueous sodium carbonate was added and the phases were mixed for 10 minutes; the phases were separated by centrifugation for 10 minutes at 1000*g*; the organic phase was washed with 2.0 mL water and then evaporated to dryness under a gentle stream of nitrogen. The dry samples were stored at −20°C until required for analysis.

The samples were redissolved in toluene (50 μL) and the substituted PFPTH of the alkylated *N*-terminal valine adduct was analyzed by GC-NECI-MS/MS with MRM on a Finnegan TSQ 700 triple-quadrupole mass spectrometer equipped with a Varian 3400 gas chromatograph. The analyses were performed by a 1-μL cold on-column injection using a DB5 MS column (30 mm × 0.25 mm; 0.25 μm film thickness; J&W Scientific) with helium as a carrier gas moving at a flow rate of 1 mL/min. The initial oven temperature was 80°C. After 1 minute, the temperature was increased at a rate of 30°C/min to 190°C and then by an increase of 3°C/min to 320°C. Methane was used as the moderator gas and argon as the collision gas at a source temperature of 120°C and a source pressure of 5000 mTorr (6.67 hPa). Ions of the derivatives at *m/z* 374 → 304 and *m/z* 374 → 318 for HBVal-PFPTH and *m/z* 380 → 304 and *m/z* 380 → 320 for [d_6]HBVal-PFPTH were measured.

MATERIALS AND METHODS FOR THBVal ANALYSIS

Synthesis of Internal Standards

The internal standard, THB[$^{13}C_5$]Val, was synthesized by Dr J Krzeminski of the American Health Foundation under contract from the National Cancer Institute Chemical Carcinogen Reference Standards Repository and further derivatized, purified, and characterized as previously reported (Swenberg et al 2000a). All other reagents and solvents used were analytic-reagent or HPLC grade.

Calibration Standards and Curves

Calibration curves were constructed by adding the internal standard, THB[$^{13}C_5$]Val-PFPTH, and analyte, THBVal-PFPTH, to 10 mg unexposed rat globin, performing the Edman degradation as described below and acetylating the THBVal adducts as described below.

QC Standards

A calibration standard of known amounts of THBVal-PFPTH and THB[$^{13}C_5$]Val-PFPTH was made and injected

as every tenth sample to ensure run-to-run reproducibility. Corrective action was taken if any significant change occurred in the relative response factor.

QC Procedures

The GC-ECNCI-HRMS instrument was calibrated with perfluorokerosene before the quantitative analysis of each set of samples. A calibration standard was injected as every tenth sample to check for run-to-run reproducibility.

THBVal Analysis

The methods used to measure THBVal adducts in coded samples in this study have been reported in detail previously (Swenberg et al 2000a). The study analyzed THBVal in coded samples. Globin was isolated according to the method of Mowrer and associates (1986). Briefly, washed red cells were lysed with an equal volume of distilled deionized water and then 9 mL of 50 mM HCl in 2-propanol was added for each milliliter of red cells. This solution was centrifuged at 1500*g* for 30 minutes at 4°C. The pellet was discarded and to the supernatant was added 4 mL ethyl acetate for each milliliter of red cells. This solution was centrifuged at 1500*g* for 5 minutes at 4°C. The globin was washed four times with ethyl acetate. The pellet was washed one more time with *n*-pentane, centrifuged as described above, and the pellet was dried under nitrogen; it was further dried under vacuum and then frozen at −80°C until analyzed.

The *N*-alkyl Edman degradation procedure of Törnqvist and coworkers (1986) was used with further modifications of Pérez and associates (1997) and Swenberg and colleagues (2000a). Briefly, 50-mg to 250-mg samples were subjected to Edman degradation in 3 mL formamide with 20 μL PFPITC (Törnqvist et al 1986). The internal standard, THB[$^{13}C_5$]Val-PFPTH, was added after Edman degradation, the mixture was heated to 45°C for 90 minutes, and the samples were extracted three times with ether, dried under nitrogen, and dissolved in 1 mL of 0.1 M sodium carbonate. The samples were then loaded onto prepared C_{18} columns (which had been prewashed with methanol and 50% aqueous formamide), washed with water, and the THBVal was eluted with 3 mL acetonitrile. The acetonitrile was dried; the residue was then resuspended in 188 μL acetonitrile and acetylated with 32.5 μL triethylamine and 32.5 μL acetic anhydride for 30 minutes at room temperature. The samples were dried under vacuum, resuspended in 3 mL *n*-pentane, and washed with 40% aqueous methanol. The aqueous layer was removed and the solution was dried under vacuum and reconstituted in 50 μL toluene.

Samples were analyzed by GC-ECNCI-HRMS (Ranasinghe et al 1998) with methane as the reagent gas (3×10^{-5} mbar), using a VG 70-250SEQ hybrid mass spectrometer with a Hewlett-Packard 5890 gas chromatograph. The GC separation was performed on an Alltech SE-54 column (30 mm × 0.32 mm; 1.0 μm film thickness; Alltech Associates, Deerfield IL) with helium at 10 psi head pressure and 300°C injector temperature. Injections were made in the direct mode starting at 100°C and increasing to 300°C at the rate of 10°C/min. The instrument was operated at 70 eV and emission current at 500 μA. The source temperature was 250°C during THBVal analysis. Quantitative selected ion monitoring measurements using the ions [M—HF] at *m/z* 534.1084 and *m/z* 539.1254 were performed at a resolving power of 10,000.

STATISTICAL ANALYSES

A complete description of all statistical methods is presented in the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Data Processing and Statistical Analysis.

RESULTS

HBVal Adduct Concentrations

With the methods used, the LOD for HBVal in human blood was approximately 0.1 pmol/g globin. This method applied [d_6]HBVal as the internal standard, which was calibrated against [^{14}C]HBVal of known specific radioactivity (Richardson et al 1998c, 1999). Using [d_6]HBVal as the internal standard produced fivefold higher concentrations of HBVal compared with the concentrations other researchers obtained when using [d_4]-(2-hydroxyethyl)valine as the internal standard (Osterman-Golkar and Bond 1996; Richardson et al 1996).

Results of HBVal concentrations in blood by exposure group are given in Table 23. The mean adduct concentration was 0.224 pmol/g globin for the control group, 0.466 pmol/g globin for the monomer group, and 2.230 pmol/g globin for the polymer group. The differences in mean concentrations parallel the differences in BD exposure levels: polymerization workers > monomer production workers > administrative control subjects. Furthermore, each group mean differed significantly from every other group's mean (Kruskal-Wallis test; $P < 0.001$).

Genotypes did not significantly affect the mean HBVal adduct concentrations, either overall or within any exposure group (data not shown). In the control group, adduct levels for smokers were significantly higher than for nonsmokers ($P = 0.027$ by Mann-Whitney test). The 97.5th percentile HBVal in the control group was 0.61 pmol/g globin (data not shown). Therefore, monomer production

Table 23. Hemoglobin Adducts[a] by Exposure Group

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)
HBVal			
Mean ± SD	0.224 ± 0.205	0.466 ± 0.452[b]	2.230 ± 1.399[b,c]
Median	0.164	0.272	1.877
THBVal			
Mean ± SD	94.77 ± 38.71	178.73 ± 101.31[b]	716.70 ± 425.72[b,c]
Median	78.70	142.60	605.65
Ratio THBVal/HBVal			
Mean ± SD	599.9 ± 326.8	540.0 ± 289.5	367.8 ± 191.6[b,c]
Median	568.9	473.9	302.2

[a] Adducts were measured as pmol adduct/g globin.

[b] Significantly different from control group at $P < 0.05$ by Kruskal-Wallis test.

[c] Significantly different from monomer group at $P < 0.05$ by Kruskal-Wallis test.

and polymerization workers who had HBVal concentrations that exceeded this value were most likely occupationally exposed to BD.

HBVal concentrations were also significantly correlated ($P < 0.001$) with workers' average BD concentrations over the entire exposure assessment period, as well as with the average of only those exposure measurements obtained during the period 70 to 50 days before blood sampling. The Spearman correlation coefficients were 0.676 for the entire assessment period average BD exposure and 0.648 for the 70–50-day assessment period average BD exposure. Figure 16 shows the relation between HBVal adduct concentrations and average BD exposure levels over the entire assessment period. The gap to the right of the lowest BD values is an artifact of the LOD of the personal monitors. Six administrative control subjects with measured exposures of zero were assigned values of 0.002 mg/m^3, which is half the BD LOD of approximately 0.004 mg/m^3. Because this value is below reported environmental levels of BD, their actual values were probably higher. When these points were excluded, the Pearson correlation between ln(*adduct concentration*) and ln(*average BD exposure*) was 0.700 (R^2 = 0.491). This relation is shown by the equation:

$$\ln(\textit{HBVal in pmol/g globin}) = 0.098 + 0.491 \times (\ln[\textit{average BD exposure in mg/m}^3])$$

which is equivalent to

$$\textit{HBVal in pmol/g globin} = 1.101 \times (\textit{average BD exposure in mg/m}^3)^{0.491}.$$

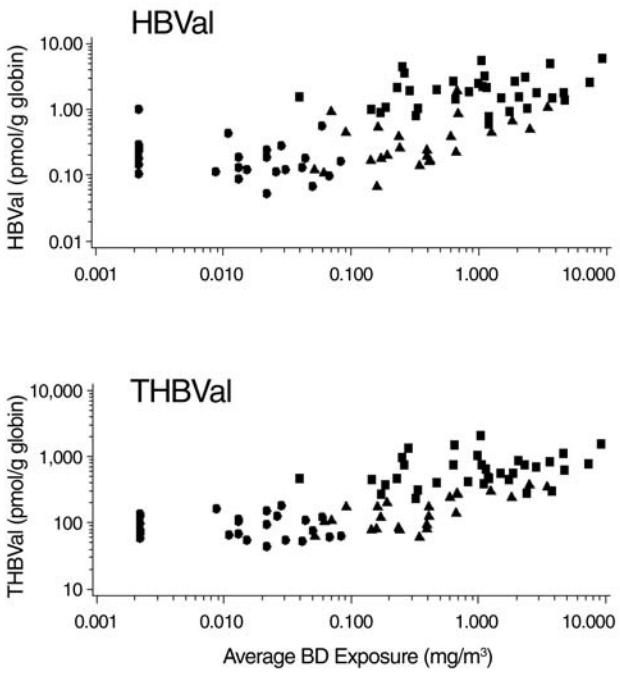

Figure 16. HBVal or THBVal Hb adducts vs average BD exposure. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

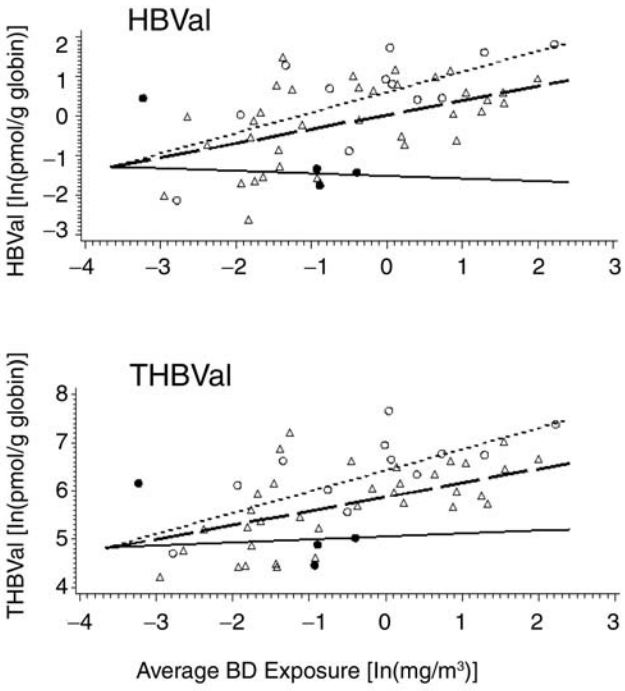

Figure 17. HBVal or THBVal Hb adducts vs average BD exposure by workers with the *EH139* genotype. ● and solid line indicate workers with *Arg139/Arg139* genotype; ○ and short-dashes line indicate workers with *Arg139/His139* genotype; △ and long-dashes line indicate workers with *His139/His139* genotype. Slope for *Arg139/Arg139* (solid line) differs significantly from slopes for *Arg139/His139* (short-dashes line) (HBVal: $P = 0.004$; THBVal: $P = 0.002$) and *His139/His139* (long-dashes line) (HBVal: $P = 0.030$; THBVal: $P = 0.035$). Slopes for *Arg139/His139* and *His139/His139* also differ significantly (HBVal: $P = 0.041$; THBVal: $P = 0.008$).

HBVal Adducts and Genotypes

As noted, worker genotype did not influence group mean HBVal adduct concentrations. However, regression analyses on BD exposure by genotype showed that workers homozygous for the *Arg139* allele of the *EH139* polymorphism (*Arg139/Arg139*) had significantly lower HBVal adduct concentrations relative to their BD exposure levels than did the workers who had *His139/Arg139* or *His139/His139* genotypes (Figure 17). The workers with the *His139/His139* genotype also had significantly lower adduct concentrations relative to the BD exposure levels than did the workers with the *His139/Arg139* genotype. The *Arg139* allele is reported to have 25% increased function over the *His139* allele (Hassett et al 1994).

THBVal Adduct Concentrations

With the methods used, the LOD for THBVal in globin was approximately 1 pmol/g globin. Mean concentrations of THBVal by exposure group are given in Table 23. As shown, the mean was 94.77 pmol/g globin for the control group, 178.73 pmol/g globin for the monomer group, and 716.70 pmol/g globin for the polymer group. This rank order again follows the same order as the mean BD exposure levels for each group. The THBVal mean concentration for each group differed significantly from each other group's mean (Kruskal-Wallis test; $P < 0.001$); and the THBVal concentrations are 321 to 423 times greater than the mean HBVal concentrations. The 97.5th percentile of the 95% CI for THBVal in the control group was 192 pmol/g globin. Therefore, monomer production and polymerization workers with THBVal concentrations that exceeded this value were most likely occupationally exposed to BD. As we found with HBVal, genotype produced no significant effect on mean adduct concentrations; smokers in the control group, however, had higher concentrations of THBVal than nonsmokers ($P = 0.011$ by Mann-Whitney test).

Again as we found with HBVal, the correlations between workers' THBVal concentrations and their average BD exposure levels were highly significant ($P < 0.001$) both over the entire exposure assessment period and during the period from 70 to 50 days before blood sampling. The Spearman correlation coefficients were 0.737 for the entire assessment period average exposure and 0.731 for the 70–50-day assessment period average exposure. The relation between THBVal concentration and average BD exposure is depicted in Figure 16. Excluding workers with exposures below the LOD, the Pearson correlation between ln(*adduct concentration*) and ln(*average BD exposure*) was 0.718 ($R^2 = 0.515$). This relation is shown by the equation:

$$\ln(\textit{THBVal in pmol/g globin}) = 6.01 + 0.395 \times (\ln[\textit{average BD exposure in mg/m}^3]),$$

which is equivalent to

$$\textit{THBVal in pmol/g globin} = 409 \times (\textit{average BD exposure in mg/m}^3)^{0.395}.$$

THBVal Adducts and Genotypes

As we found with the HBVal adducts, regression analysis of THBVal adduct concentrations on BD exposure levels by genotype showed that workers with the *Arg139/Arg139* genotype of the *EH139* polymorphism had significantly lower THBVal adduct concentrations relative to their BD exposure levels than did workers who had the *His139/His139* or *His139/Arg139* genotype (Figure 17). However, the workers with the *His139/Arg139* genotype also had significantly greater THBVal adduct concentrations relative to their BD exposure levels than did the workers with the *His139/His139* genotype.

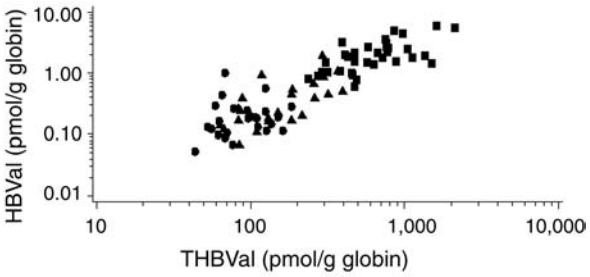

Figure 18. Relation between HBVal and THBVal Hb adducts. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

Correlation Between HBVal and THBVal

The concentrations of HBVal and THBVal in blood were highly and significantly ($P < 0.001$) correlated. The Spearman correlation coefficient was 0.853. The Pearson correlation was 0.879 ($R^2 = 0.773$) when the natural logarithms of both adducts were used (Figure 18). This relation is shown by the equation:

$$\ln(\textit{HBVal in pmol/g globin}) = -6.57 + 1.101 \times \ln(\textit{THBVal in pmol/g globin}),$$

which is equivalent to

$$\textit{HBVal in pmol/g globin} = 0.00140 \times (\textit{THBVal in pmol/g globin})^{1.101}.$$

Ratio of THBVal to HBVal

THBVal/HBVal ratios were computed for each worker and the mean ratios for the control, monomer, and polymer groups are given in Table 23. THBVal concentrations for the polymerization workers were 368-fold higher than HBVal concentrations; this ratio was significantly lower than the 540-fold ratio for the monomer production workers, and the 600-fold ratio for the administrative control subjects (Kruskal-Wallis test; $P = 0.003$). We also noted an inverse relation between subjects' THBVal/HBVal ratios and their average exposure concentrations ($r = 243$; $P = 0.027$). This was due to the endogenous background of THBVal, which contributes more to the lower exposures.

DISCUSSION

The procedure for measuring HBVal, the Hb adduct of BDO, as a biomarker of BD exposure was developed in the early nineties (Osterman-Golkar et al 1991) and values have been reported for rodents and humans (Osterman-Golkar et al 1993; van Sittert and Van Vliet 1994; Osterman-Golkar and Bond 1996). In the present study, the commonly used internal standard, $[d_4]$-(2-hydroxyethyl)valine, was replaced by $[d_6]$HBVal to improve the accuracy and the reliability of the method. Using $[d_6]$HBVal as an internal standard suggests that previously published values for HBVal in Hb (which had been determined using $[d_4]$-(2-hydroxyethyl)valine as the internal standard; Osterman-Golkar et al 1991, 1993; Osterman-Golkar and Bond 1996; Richardson et al 1996) were most likely underestimated by a factor of 5. In the current study, the natural background levels of HBVal adducts were very low, ranging from below the LOD (0.1 pmol/g globin) to 1.0 pmol/g globin in administrative control subjects who were not occupationally exposed. The measurement techniques for THBVal, the Hb adduct of BDO-diol, were developed more recently (Pérez et al 1997) and have been successfully applied in an epidemiologic study (Swenberg et al 2000a; Hayes et al 2000).

Both HBVal and THBVal were successful as biomarkers for BD exposure because the average values for each adduct in the three exposure groups ranked in the same order as the exposure levels for the same groups. In addition, we found highly significant correlations between the individual subjects' exposure levels and both types of Hb adducts. Linear regression models of the relation between BD exposure (average 60 days) and HBVal or THBVal in Hb fitted best following logarithmic transformation of the variables. This demonstrates that the proportion of inhaled BD that binds with Hb to form HBVal or THBVal decreases at increasing BD exposures. From the regression equations, HBVal and THBVal adduct levels in Hb can be calculated for specific BD airborne exposures. For instance, an 8-hour TWA exposure of 22 mg/m^3 (10 ppm) corresponds to about 5 pmol HBVal/g globin and to 1400 pmol THBVal/g globin.

The finding of relatively high natural background levels of the M1 urinary metabolite of BD and of THBVal adducts in Hb from individuals with no known exposures to BD (the administrative control subjects in this study) demonstrates that THBVal is not only produced by exposure to BD but also by other sources. Results of an epidemiologic study (Hayes et al 2000), in which THBVal was determined by the same method as in the current study, suggest that the naturally occurring background of THBVal is variable: The median background THBVal value for unexposed workers in the Hayes study was 38 pmol/g globin, which is much lower than the median value determined in the present study (79 pmol/g globin). After BD exposure, THBVal adducts are formed by the reaction of BDO_2 or BDO-diol (or both) with Hb. (BDO-diol can be formed either by hydrolysis of BDO_2 or by CYP-mediated oxidation of BD-diol [Csanády et al 1992; Kemper et al 1996].) It is unlikely that background levels of THBVal originate solely from BDO_2 or BDO-diol arising primarily as metabolites from environmental or exogenous BD exposure.

Endogenous THBVal may also originate from one or more endogenous chemicals that are presently unknown. The data from both the current study and the study by Hayes and associates (2000) suggest that other factors such as diet or genetics affect the endogenous production of THBVal. The implications of these natural background levels for cancer risk of BD exposure are currently unknown. However, THBVal may be less sensitive than HBVal for detecting low levels of BD exposure due to the relatively high and variable background levels.

Genotypes for *CYP2E1*, *GST*, *EH*, and *ADH* polymorphisms were obtained for all workers involved in this study. Although the *CYP2E1*, *GST*, and *ADH* polymorphisms did not show associations with Hb adducts, one of the *EH* polymorphisms did. Workers with *Arg139/Arg139* in the *EH* gene (an allele with increased activity) had significantly lower HBVal and THBVal adduct concentrations relative to BD exposure levels than did workers with the other two genotypes of this polymorphism. In addition, the workers with the *His139/Arg139* genotype actually had greater concentrations of both adducts compared with workers with the other two genotypes. The decreased concentration of HBVal adducts derived from the BDO metabolite of BD might be explained by an increase in hydrolysis of BDO in workers with the *Arg139/Arg139* genotype. This does not explain, however, that HBVal adduct production was greater in workers with the *His139/Arg139* genotype than in workers with the *His139/His139* genotype. Other observations are also not in accord with this rationale; for example, the increased concentrations of the THBVal adducts in workers with the *His139/Arg139* genotype and in workers with the *His139/His139* genotype require a different explanation because THBVal adducts are thought to derive primarily from the BDO-diol metabolite of BD, production of which is dependent on the hydrolytic pathway (see Figures 1, 2, and 15). These data suggest that the *EH* genotypes have differential kinetics for the three epoxide metabolites of BD. The increased production of THBVal adducts in workers with the *His139/His139* genotype and with the *His139/Arg139* genotype might reflect the small proportion of the THBVal adducts that are derived from the BDO_2 metabolite of BD. This does not explain, though, that the THBVal adduct production in workers with the *His139/Arg139* genotype was significantly greater than in workers with the *His139/His139* genotype. However, the numbers and distributions of the workers with different *EH139* genotypes in this study are far too small to permit definitive assessments of these possibilities (especially because only four subjects had the *Arg139/Arg139* genotype). Nevertheless, the current study does provide genotypic data that may be combined with findings from other molecular epidemiologic studies to suggest further animal studies in rodents genetically modified in these metabolic pathways.

The mean THBVal adduct levels in each exposure group were manyfold higher than the mean HBVal levels in the same group: control group 600-fold higher, monomer group 540-fold higher, and polymer group 368-fold higher. This suggests that THBVal is also being formed by an endogenous material other than BD and its metabolites. This endogenous THBVal is likely to be relatively constant, so the ratios change as BD-derived THBVal increases. It may be more correct to subtract the control value for THBVal from each of the exposed values and then use the ratio BD-THBVal:BD-HBVal. (The same effect has also been observed in mice but not in rats [Richardson et al 1997, 1998a].)

The current study has demonstrated that both of these Hb adducts can be used as biomarkers to monitor groups of workers for low levels of exposure to BD. The excellent correlation between exposure measurements and these Hb adducts also permits exposure ranking of individuals for nonspecific biomarkers of genetic effects such as somatic mutations and cytogenetic changes. This permits researchers to examine exposure-response relations within the exposed population and to eliminate confounding by problems that arise in control groups. Such exposure ranking was used in the comparative studies of genetic effects in this investigation (see the later sections Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes; and Biomarkers of Effect: Cytogenetic Analysis). By combining exposure ranking on the basis of biomarker assessments with the requirement that all assays had to be completed before the exposure coding of the individual samples was revealed, this study was conducted with a high degree of scientific rigor.

BIOMARKERS OF EFFECT: ASSAYS OF *HPRT* MUTATIONS IN HUMAN LYMPHOCYTES

An obvious question is whether occupational exposure to BD could induce mutations in exposed workers. Because BD is mutagenic in animals and in cells in culture, it seems logical that exposure at some level would cause mutations in humans. A useful biomarker of human exposure to mutagens is the assay for mutations at the *HPRT* gene in T lymphocytes. An increased frequency of mutant lymphocytes in members of a population exposed to a known mutagen would suggest a possibly hazardous situation that warrants further investigation. This type of biomarker would constitute an early warning of a potential health hazard, thus providing an opportunity to institute measures to reduce future exposures and possibly prevent exposure-related illness.

The frequency of mutations at the *HPRT* gene can be measured in two ways: the cloning assay and the autoradiographic assay (Albertini 1985; O'Neill et al 1987; Ammenheuser et al 1988). In both methods, mutant cells are selected by growing T lymphocytes in the presence of a purine analogue, 6-thioguanine (TG). The cloning assay for *HPRT* mutations involves growing clones of TG-resistant lymphocytes cultured in microwell plates over a period of 10 to 14 days. This permits the mutant frequency (MF; the frequency of mutant lymphocytes) in the peripheral blood to be determined and provides a source of viable mutant cells from which DNA and RNA can be obtained for molecular analysis to ascertain the nature of the *HPRT* mutations. In the autoradiographic version of the assay, TG-resistant cells are selected on the basis of their ability to incorporate tritiated thymidine ([^{3}H]TdR) during a 42-hour culture period in the presence of TG. Radiolabeled mutant cells are then identified on microscope slides by autoradiography. This method does not provide viable cells for further analysis, so mutant cells are referred to as variants. The assay is very suitable, however, for analyzing relatively large numbers of samples. In addition, the assay has proven to be a sensitive biomarker for mutagen exposure. For example, the baseline variant frequency (VF) in nonsmokers who have no other known mutagen exposure has been consistently measured in the range of 1 to 4 variants per million viable cells (Ammenheuser et al 1997b, 1998).

Somatic cell mutation has several unique strengths as a biomarker of the effects of human exposure to genotoxic agents. First, mutation is the end product of the mutagenesis process. The induction of mutations by a chemical exposure integrates the effects of biotransformation, DNA damage, DNA repair, and cytotoxicity. Second, because mutations are heritable, they represent a type of toxic effect that, if it occurs in specific genes, can lead to diseases such as cancer. In contrast, unstable chromosomal aberrations represent a form of DNA damage that may be eliminated by cell death. Third, somatic mutation assays, such as the *HPRT* assay in lymphocytes, respond well to mutagens that generate base substitution, frameshift, or deletion mutations. In many cases the *HPRT* assay is complementary to assays that detect large-scale chromosomal aberrations (Ammenheuser et al 1991; Tates et al 1996). The *HPRT* cloning assay provides an opportunity to further examine the mechanisms of mutation associated with an exposure by evaluating the spectrum of the types of mutations that have been induced (Nicklas et al 1990; Hüttner and Holzapfel 1996; Ma et al 2000).

Because mutations are the end product of the mutagenesis process, they are nonspecific to the causes. For example, many exposures encountered in the course of daily living, such as certain medications, tobacco smoke, or the use of drugs such as cocaine or marijuana may influence the frequency of mutations (Ammenheuser et al 1988, 1997a,b, 1998); even age gradually increases mutation frequency (Tates et al 1991b, Robinson et al 1994). Factors that influence the in vivo proliferation of lymphocytes may also increase mutation frequency. For example, cycling lymphocytes are more susceptible to mutation, so autoimmune diseases or other processes that stimulate lymphocyte proliferation could increase the observed MF (Cannons et al 1998). In addition, proliferation of a mutant lymphocyte will produce a clone of identical mutants, which can also increase the MF (Nicklas et al 1989). It is therefore important in studies of mutagen exposures to obtain accurate measurements of exposure and to control as much as possible for other factors that may influence the MF.

Workers exposed to BD have been investigated for the frequency of *HPRT* mutants in several studies with conflicting results. In an early study (Ward et al 1994), a small cohort of nonsmoking workers in a BD monomer production plant in Port Neches, Texas, was evaluated. Using the autoradiographic assay, workers in high-exposure production units had a significant threefold elevation ($P < 0.01$) in *HPRT* VF relative to workers in low-exposure areas and unexposed control subjects. No concurrent measurements of BD exposure were made in this study, but the average BD levels were estimated to be 2 to 7 mg/m^3 (1–3 ppm) based on an exposure study conducted by the company about 3 to 9 months prior to sample collection (Ward et al 1994). Furthermore, the concentration of the BD-related metabolite urinary M1 was determined and levels of this BD metabolite correlated well with the *HPRT* VF ($r = 0.85$). This study generated a great deal of interest in the question of the potential mutagenic effects of occupational exposure to BD (OSHA 1996).

A follow-up study was conducted in the same facility about eight months after the first study (Ward et al 1996b). During this interval, a modernization project had been completed that included several measures intended to reduce workers' exposures to BD. Concurrent BD measurements were made and the *HPRT* VF and urinary M1 levels were again determined in a somewhat larger group of workers. In this second study, workers were divided into three exposure categories and exposures were lower than in the earlier survey: high (0.66 mg/m^3 [0.30 ppm]), intermediate (0.46 mg/m^3 [0.21 ppm]), and low (0.27 mg/m^3 [0.12 ppm]). The urinary M1 concentrations for high-exposure subjects were reduced to levels slightly higher than the concentrations for subjects with low BD exposures. The *HPRT* VF was still significantly elevated ($P < 0.01$), but the increase in the high-exposure group was now only about 2.5-fold over the low-exposure group (Ward et al 1996b).

Two larger biomarker studies were conducted, about three years apart, in a BD-styrene polymer production plant, also in Port Neches, Texas. In both studies (Ammenheuser et al 2001; Ward et al 2001), workers in areas of the plant with higher and lower exposures to BD were compared. In the first study, concurrent exposures were measured with the workers wearing passive badge dosimeters that provided a minimum LOD of about 0.55 mg/m^3 (0.25 ppm) for an 8-hour workshift. The *HPRT* VF was measured by autoradiography and levels of urinary M1 were determined. The average exposure for 22 of the 24 workers in the high-exposure areas (two high values excluded) was 3.26 mg/m^3 (1.48 ppm), whereas all but two exposures in the low-exposure areas were below the LOD for the assay (0.55 mg/m^3). For workers from the high-exposure areas, the average urinary M1 concentration was more than three times greater and the average *HPRT* VF was almost three times greater than the same values for workers from the low-exposure areas ($P < 0.0002$). Exposure, M1, and *HPRT* VF were all correlated, with coefficients between 0.4 and 0.6 (Ammenheuser et al 2001).

A subset of samples from nonsmoking high-exposure-area workers was also assayed by the *HPRT* cloning method, and isolates were analyzed to determine the types of mutations found (Ma et al 2000). Although the number of workers evaluated in this subset was not stated, 12 had exposures above the LOD with a range of 0.55–45.97 mg/m^3 (0.25–20.80 ppm). The *HPRT* MF was about twofold greater ($P < 0.05$) in this exposed subset (MF $\pm$ SD = $17.63 \times 10^{-6} \pm 5.05 \times 10^{-6}$) than in an unexposed control group ($8.47 \times 10^{-6} \pm 2.88 \times 10^{-6}$). The proportion of single-exon and multiple-exon deletions and frameshift mutations was significantly greater in the exposed group ($P < 0.05$) than in the control group. This is of interest because cell culture and animal studies have shown that the BDO_2 metabolite of BD induces a high proportion of deletion mutations in the *HPRT* gene (Cochrane and Skopek 1994b; Meng et al 2000).

In the second study, a follow-up of 37 workers conducted three years later in the same facility, the same researchers again tested lymphocytes for *HPRT* VF, and more detailed measures of exposure and M1 were taken (Ward et al 2001). The average exposure for the high-exposure group in this study was 3.63 mg/m^3 (1.65 ppm) compared with the average exposure of 0.15 mg/m^3 (0.07 ppm) for the low-exposure group. The average *HPRT* VF was again about threefold greater in the high-exposure group compared with the low-exposure group ($P < 0.001$). In this second study, the subset of high-exposure workers who were tobacco smokers had a mean VF that was more than 1.5-fold greater than the high-exposure nonsmokers. The urinary M1 levels were higher in the high-exposure group than in the low-exposure group (Ward et al 2001), but the magnitude was not as great as in the earlier study (Ward et al 1994; Ammenheuser et al 2001).

Other studies of occupational exposure to BD have been conducted in China and in the Czech Republic. Two of these studies used the *HPRT* cloning assay to assess the mutagenic effects of BD exposure. The study conducted in China evaluated the exposure of workers in a polybutadiene rubber production facility, which included both monomer production and polymerization units. Workers (41) in three exposure areas were compared with unexposed subjects (38). Median air exposure to BD was 4.42 mg/m^3 (1.97 ppm), but this relatively high value was reported to be due largely to intermittent high exposures that were associated with activities such as pump repair. The *HPRT* MF was similar in the exposed and control groups and was not affected by cigarette smoking (Hayes et al 2000).

The study conducted in the Czech Republic (Tates et al 1996; Šrám et al 1998) evaluated workers from the same BD production facility near Prague that we studied in the current project. In 1993 and 1994, 19 BD-exposed workers in the monomer production unit were compared with 19 unexposed workers from a heat production unit. Exposures were measured using passive badge dosimeters and ranged between 0.03 and 43.69 mg/m^3 ([0.01–19.78 ppm] with a mean of 3.89 mg/m^3 [1.76 ppm]) for the exposed workers. Average BD exposure for the unexposed workers was 0.09 mg/m^3 (0.04 ppm). No significant difference was noted between exposed and control subjects in the geometric mean *HPRT* MF, adjusted for cloning efficiency (CE), smoking, and age; and no effect of smoking on MF was noted (Tates et al 1996). In the 1994 portion of that study, however, the analysis of cells for chromosomal aberrations showed a small but significant increase in the

percentage of aberrant lymphocytes in BD-exposed workers ($P < 0.01$; Šrám et al 1998). No such increases had been found in the earlier samples from these workers (Sorsa et al 1994).

Overall, studies of the mutagenic effects of occupational exposure to BD are conflicting. In the Texas studies using the autoradiographic assay, an increase in VF of around threefold was noted in workers with mean BD exposures of about 3.32 to 6.63 mg/m^3 (1.50–3 ppm). Studies in China evaluating workers with similar or somewhat higher exposures, and in the Czech Republic evaluating workers with somewhat lower exposures, found no effect of BD exposure using the *HPRT* cloning assay. The only direct comparison of the two types of *HPRT* assays was with a selected subset of workers in the first Texas SBR study (Ma et al 2000) in which workers were exposed to a median BD level of 1.90 mg/m^3 (0.86 ppm). A twofold increase in *HPRT* MF in exposed workers was found when compared with outside control subjects ($P < 0.05$); and in parallel assays, a threefold increase in VF was noted. One goal of the current study was to attempt to resolve the conflict in the results of BD exposure studies by using both types of *HPRT* assays to evaluate a significant population of workers after careful assessment of their exposure to BD.

MATERIALS AND METHODS

Cloning Assay

Receipt and Handling of Samples On 2 June 1998 at 2 PM, samples of isolated peripheral blood lymphocytes (PBLs) were sent in a dry shipper with liquid nitrogen from Prague to Leiden. The dry shipper arrived in Leiden on 5 June at 2 PM containing blood samples from 82 subjects: 25 administrative control subjects, 23 (not 24) monomer production workers, and 34 polymerization workers. (The volume of blood collected from two exposed subjects was insufficient to divide among all the laboratories performing assays; therefore, the blood from one monomer production worker was *not* sent to Leiden for the cloning assay and the blood from one polymerization worker was *not* sent to Galveston for the autoradiographic assay. Thus, in the Leiden cloning assays, the monomer group $n = 23$ rather than 24.) Each sample consisted of two ampules, except those from subjects 133 and 135 for whom just one ampule was received. The numbering of the samples in the dry shipper from Prague was in conformity with the list of samples received by mail from Prague. Upon arrival, the ampules looked normal and were transferred to a container with liquid nitrogen where they remained until they were used to screen for *HPRT* mutants with the cloning assay.

Quality Control Procedures Usually, six randomly picked Prague samples (a total of twelve ampules) were processed simultaneously in one set during a particular working week. During later statistical treatment of the results, such sets could be identified by a special code so that eventual mishaps or irregularities during the performance of the cloning assay could be easily found.

Apart from the 82 subject samples from Prague, frozen PBLs from one healthy Leiden subject were processed concurrently with each set of six Prague samples (starting with the fourth of 13 sets) and used as internal QC standards. For the QC standard PBLs, we measured nonselection CEs to monitor the capacity of T lymphocytes to grow in vitro in medium without the selection agent TG (−TG). CEs for the internal QC standards were not included in the tables; the mean ± SD CE for the QC standards was 24.08% ± 2.73% (range: 19.70–28.01%; $n = 10$ [one internal QC standard analyzed with 10 of 13 sets of Prague samples]).

Thawing Frozen Samples Ampules were thawed by gently agitating them in a water bath at 37°C. When thawing was complete, ampules were placed on crushed ice. The contents of each ampule were transferred into a prechilled centrifuge tube. Prechilled thawing medium (RPMI 1640 culture medium supplemented with 40% BCS) was added drop-wise to the cell suspension (meaning: add one drop, gently agitate, repeat a few times, then add two drops, repeat, etc). About 8 mL thawing medium was added in a time span of 20 to 30 minutes.

After centrifugation (260*g*) for 10 minutes at room temperature, the cell pellet was suspended in culture medium (see below) and the cell density counted with a Fuchs-Rosenthal hemocytometer. For the first 24 subjects (4 sets), only the number of living cells per subject was counted; later, numbers of dead and living cells were recorded. Cells were counted using phase-contrast microscopy. Dark gray cells were interpreted as being dead whereas light gray transparent cells were classified as living (Table 24).

Preincubation PBLs were preincubated for 24 hours at 1×10^6 living PBLs/mL culture medium (see below) in horizontal 50-mL or 250-mL tissue culture flasks in a humidified incubator at 37°C in an atmosphere of 5% to 6% CO_2 in air. This was done to remove monocytes, which remained attached to the flask surface.

For all subjects, the number of live cells was counted at the end of preincubation (as well as after thawing). The numbers of cells alive after thawing and alive after preincubation

Table 24. Summary of Cloning Assay Results

Parameter	Control	Monomer	Polymer
Cells alive ($\times 10^6$) after thawing			
Mean ± SD	8.92 ± 4.73	13.85 ± 6.90[a]	8.69 ± 5.20
Median (range)	7.20 (4.30–22.10)	12.50 (4.50–32.80)	7.00 (1.30–29.60)
Quartile range 25%–75%	6.00–10.70	9.30–16.45	5.40–11.48
n (= 82 total)[b]	25	23[b]	34
Cells alive ($\times 10^6$) after preincubation			
Mean ± SD	6.48 ± 3.83	11.05 ± 5.81[a]	7.03 ± 4.13
Median (range)	5.10 (2.93–19.26)	9.99 (2.80–25.50)	5.59 (1.50–23.70)
Quartile range 25%–75%	4.13–7.52	7.58–13.65	4.61–8.42
n (= 82 total)[b]	25	23[b]	34
Cells recovered (%) after preincubation[c]			
Mean ± SD	73.74 ± 16.88	78.74 ± 14.84	89.14 ± 54.51
Median (range)	79.17 (45.00–106.41)	77.74 (53.00–115.00)	81.41 (46.27–380.77)
Quartile range 25%–75%	56.58–90.00	67.39–89.40	69.15–90.54
n (= 82 total)[b]	25	23[b]	34
CE (%)			
Mean ± SD	17.51± 6.53	13.43 ± 8.22	12.82 ± 8.54
Median (range)	17.01 (4.84–31.09)	15.48 (0.44–26.46)	11.50 (0.17–30.06)
Quartile range 25%–75%	13.99–21.93	6.2–19.28	6.87–18.45
n (= 78 total)[d]	24	22	32
Clonable cells ($\times 10^6$)			
Mean ± SD	1.12 ± 0.94	1.59 ± 1.37	1.01 ± 1.06
Median (range)	0.88 (0.21–3.86)	1.29 (0.03–5.04)	0.62 (0.03–3.82)
Quartile range 25%–75%	0.54–1.29	0.36–2.58	0.32–1.33
n (= 78 total)[d]	24	22	32
MF ($\times 10^{-6}$) for all subjects			
Mean ± SD	13.01 ± 8.10	9.24 ± 6.24	18.83 ± 17.41
Median (range)	9.90 (2.53–35.21)	8.34 (0.00–22.70)	13.84 (3.34–83.55)
Quartile range 25%–75%	6.98–18.97	6.05–13.53	7.14–28.08
n (= 78 total)[d]	24	22[e]	32
MF ($\times 10^{-6}$) for smokers			
Mean ± SD	13.24 ± 6.19	12.10 ± 6.10	14.99 ± 10.77
Median (range)	9.96 (5.51–20.13)	10.57 (0.00–22.70)	13.84 (4.55–45.38)
Quartile range 25%–75%	9.58–18.98	8.37–16.03	7.95–15.56
n (= 35 total)[f]	9[f]	12[e]	14
MF ($\times 10^{-6}$) for nonsmokers			
Mean ± SD	13.34 ± 9.42	5.81 ± 4.62	21.83 ± 21.03
Median (range)	10.10 (2.53–35.21)	6.47 (0.00–14.31)	12.22 (3.34–83.55)
Quartile range 25%–75%	7.30–17.30	2.12–7.74	7.11–33.18
n (= 42 total)[f]	14[f]	10[e]	18

[a] Significantly different ($P = 0.003$) from the other two groups by Kruskal-Wallis test.

[b] One monomer production worker's blood sample was not shipped from Prague to Leiden for analysis; therefore, for total subjects, $n = 82$ rather than 83, and for the monomer group, $n = 23$ rather than 24.

[c] Calculated as: [cells alive after preincubation / cells alive after thawing] × 100.

[d] Due to unsuccessful cloning, 4 samples were lost: 1 nonsmoker in the control group, 1 smoker in the monomer group, and 2 smokers in the polymer group. Therefore, total $n = 78$ instead of 82 (number of samples shipped from Prague) and group n values change accordingly.

[e] Data for the three monomer production workers (1 smoker and 2 nonsmokers) with MF = 0 were included to calculate the mean, median, and quartiles. These subjects were automatically excluded from further analysis of the data using log-transformed MFs because log = 0 does not exist.

[f] Data from 1 administrative control subject were omitted due to uncertain smoking status. Therefore, for those analyses involving smokers vs nonsmokers, the total number in the control group was 23 rather than 24.

were used to calculate the percentage of cells recovered after preincubation [(cells alive after preincubation / cells alive after thawing) × 100] (Table 24).

Culture Medium Culture medium consisted of RPMI 1640 (Dutch modification, Gibco) with 25 mM HEPES, 2 g/L sodium bicarbonate ($NaHCO_3$), and 2 mM L-glutamine (Gibco); supplemented with 20% AIM-V serum-free medium (Gibco), 15% heat-inactivated DBS (iron-supplemented defined BCS; Hyclone, Logan UT), 1% β-mercaptoethanol (final concentration 50 μM), 1% L-glutamax (Gibco; final concentration 2 mM), 1% sodium pyruvate (Gibco; final concentration 1 mM), and antibiotics 1% (final concentration 100 units/mL penicillin and 100 μg/mL streptomycin sulfate).

The culture medium was freshly prepared in bulk for each set of six randomly selected Prague samples. By following this procedure we excluded the possibility that a mistake in preparing the culture medium would result in an accidental failure to measure values for CE or MF or both for a particular subject.

Cloning Lymphocytes We cloned PBLs in 96-well round-bottom microtiter plates (Nunc-Roskilde, Denmark). For each subject, two plates without TG with 3 preincubated PBLs/well, plus two plates with 6 cells/well were used to measure the mean CE (%) in nonselection medium. The number of PBLs per well pertains to the number of living cells per well regardless of whether they were stimulated [or activated (lymphoblastoid)].

In addition, each well contained 1×10^4 irradiated TK6 feeder cells. Feeder cells were *HPRT*$^-$ TK6 36X4 lymphoblastoid cells (gift from RJ Albertini). The growth of TK6 cells was arrested by irradiation with 40-Gy 200-kV 4-mA x-rays (filtering 1 mm Al) at a dose rate of 15 Gy/min (Andrex 225, Andrex Radiation Products, Copenhagen, Denmark). For selecting mutant cells, 1 to 10 +TG-selection plates (depending on the number of PBLs available for a particular subject) with 2×10^4 preincubated PBLs per well and 1×10^4 irradiated TK6 feeder cells per well were used. Each well, for both CE and selection plates, contained 200 μL culture medium supplemented with phytohemagglutinin (PHA) (PHA HA16; Wellcome Diagnostics, Murex, Dublin, Ireland) at a final concentration of 0.25 μg/mL and recombinant interleukin-2 (IL-2; EuroCetus, CHIRON, Emeryville CA) at a final concentration of 100 U/mL. The selection plates also contained TG (Sigma) at a final concentration of 2.5 μg/mL (1.5×10^{-5} M). After incubation in a humidified incubator at 37°C in an atmosphere of 5% to 6% CO_2 in air for 10 to 14 days, plates were scored for colony growth using an inverted microscope.

As described above, TK6 cells were added to the PBLs in −TG and +TG plates, but they were also seeded in a plate without PBLs. This was done to verify that irradiated TK6 cells could not form clones. TK6 cells were maintained in RPMI 1640 medium supplemented with 10% DBS, 1% β-mercaptoethanol (50 μM) and antibiotics at final concentrations of 100 units/mL penicillin and 100 μg/mL streptomycin sulfate. Cell densities ranged from 1×10^5 to 1×10^6 cells/mL and subculturing occurred thrice weekly. The cells were diluted to 0.5×10^6 cells/mL 24 hours prior to use in the cloning assay.

Scoring Clones and Calculating CEs and MFs Wells carrying clones consisting of at least 50 healthy-looking cells were recorded as positive wells. For each subject, we counted (1) the number of positive and negative wells in −TG plates and (2) the number of positive wells (equal to the number of mutants) among the total number of wells in +TG plates. This information was used to calculate for each subject: (1) CE (%) for 3 and 6 cells per well, (2) mean CE (%), and (3) MF ($\times 10^{-6}$) plus 95% CI. CEs were calculated on the basis of a Poisson distribution using the formula (O'Neill et al 1987)

$$CE = (-\ln P_0)/N,$$

where P_0 is the fraction of negative wells and N is the average number of cells plated per well. Thus:

$$CE = -\ln(Neg/Tot)/N$$

where *Neg* and *Tot* represent the numbers of negative wells or total wells, respectively. MF is the ratio of the CE in the presence and absence of TG, or CE^{+TG}/CE^{-TG}. The variance, *Vi*, was calculated with the formula

$$(1 - Neg/Tot)/\{Neg \times [\ln(Neg/Tot)]^2\}.$$

The 95% CI for MF was determined with the equation

$$CI = MF \times \exp\left(\pm 1.96 \times \sqrt{\{[Vi(-TG) + Vi(+TG)/4]\}}\right).$$

All calculations were performed using Excel spreadsheets.

Autoradiographic Assay

Receipt and Handling of Samples Cryopreserved lymphocyte samples and plasma samples in separate shipping containers with liquid nitrogen were sent from Prague, Czech Republic on 2 June 1998 to Galveston, Texas, USA. United States Customs detained the shipment for several days until

additional information about the contents was received from Prague. We received the two nitrogen shippers at the laboratory on 10 June 1998; they contained 82 samples, each of which consisted of three cryotubes per subject. (The volume of blood collected from two exposed subjects was insufficient to divide among all the laboratories performing assays; therefore, the blood from one monomer production worker was *not* sent to Leiden for the cloning assay and the blood from one polymerization worker was *not* sent to Galveston for the autoradiographic assay. Thus, in the Galveston autoradiographic assays, the polymer group $n = 33$ rather than 34.) The cryotubes containing lymphocytes appeared to be adequately frozen; but some of the plasma, in 15-mL tubes, had begun to thaw in the second shipper. The lymphocyte samples were quickly sorted, placed on canes, and stored in a Dewar flask with liquid-nitrogen. The plasma was refrozen and stored in a freezer at −20°C.

Quality Control Procedures During the same period when we cultured and analyzed the samples from subjects in Prague, we also thawed and assayed frozen lymphocyte samples (two to four per batch of Prague samples) to serve as internal QC standards. Those cells (27 additional samples) were from BD-exposed subjects in a study being conducted at a Texas SBR plant or from nonexposed Texas subjects who had volunteered for other studies conducted in our laboratory.

Thawing Frozen Samples Each sample of three cryotubes was thawed quickly in a 37°C water bath by shaking the tubes as the lymphocyte suspension thawed. The cells were diluted in 30 mL of RPMI 1640 medium (Gibco) in a 50-mL centrifuge tube by adding the suspension drop-wise using a 1-mL pipette. The diluted cells were centrifuged at 250*g* for 10 minutes, and the pellet from each sample was resuspended in 25 mL of medium. The lymphocytes were counted in a hemacytometer and viability was determined with trypan blue stain.

Culture Medium The complete medium for the autoradiographic *HPRT* assay was prepared with RPMI 1640 containing 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Gibco, Gaithersburg MD), together with a final concentration of additives consisting of 20% HL-1 medium supplement (Bio-Whittaker, Walkersville MD), 2% PHA (reagent grade; Murex, Dartford, England), and 25% autologous plasma.

Incubating Samples for* HPRT *Variant Selection Methods for culturing lymphocytes to determine *HPRT* VFs were essentially the same as previously described (Ammenheuser et al 1991, 1997b). After viable cells were counted, the cell suspension was centrifuged at 250*g* for 10 minutes and the cell pellet was resuspended in culture medium. Lymphocytes ($5–6 \times 10^6$) were added to each vented flask (Falcon 3108, Lincoln Park NJ) and TG (Sigma, St Louis MO) was added to a final concentration of 33.4 μg/mL (2×10^{-4} M). The final cell density was 1×10^6 per mL. One labeling index (LI) flask was prepared without TG but with medium adjusted to the same pH as the +TG medium. After incubation in a humidified 5% CO_2 atmosphere for 24 hours at 37°C, all cultures were labeled with 25 μCi of [^{3}H]TdR (ICN, Costa Mesa CA) and incubated for an additional 18 hours. Then the cells were harvested and free nuclei were released by the addition of 9 mL of chilled 0.1 M citric acid to each flask. Usually, eight to twelve samples were cultured at 3-week to 4-week intervals.

Fixing Cells and Preparing Slides The free nuclei from the +TG flasks were washed with fixative (methanol:acetic acid, 7:1.5) and resuspended in 1 or 2 tubes in 0.25 mL of fixative. The nuclei from the LI flask were harvested separately using the same procedure. A 20-μL aliquot from each tube from the +TG cultures was counted with a particle counter (Coulter, Hialeah FL) and all of the nuclei remaining in the tube were placed on an 18-mm × 18-mm coverslip previously affixed to the end of a microscope slide. For the nuclei from the LI flask, an aliquot of about 0.15×10^6 cells per slide was spread evenly onto two separate 18-mm × 18-mm coverslips affixed to slides. The slides were stained with aceto-orcein (Gurr BDH, Poole, England), dipped in NTB-2 emulsion (Kodak, Rochester NY), stored for 2 to 3 days in light-tight boxes at 4°C, and developed with D-19 (Kodak).

Scoring Slides and Calculating VFs Coded slides were read with a Nikon Labophot microscope, and all labeled (variant) cells from the +TG cultures were counted. For the LI slide, a random differential count was made of 3000 labeled and unlabeled cells to provide an estimate of the proportion of lymphocytes that were able to grow in culture; this proportion of labeled-to-unlabeled cells is referred to as the LI. The VF was calculated by totaling the labeled (variant) cells identified on the slides prepared from the +TG cultures and dividing this total by the LI multiplied by the number of nuclei initially added to the TG slides. The denominator for this calculation is referred to as the number of evaluatable cells (ECs).

Duplicate Scoring After the slides prepared from the +TG-selection cultures were scored in Galveston, they were packed in slide boxes and shipped to Burlington. The slides were scored again by a slide reader who was unaware of the results of the scoring in Galveston.

Statistical Analysis of Cloning and Autoradiographic Assay Data

For reasons outlined in the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Data Processing and Statistical Analysis, the data on MFs, VFs, and other relevant parameters for the cloning and autoradiographic assays were predominantly analyzed with nonparametric tests. The Kruskal-Wallis test was used to test for differences among the three exposure groups. The Bonferroni-adjusted Mann-Whitney test was applied to make pair-wise comparisons between exposure groups (control vs monomer, control vs polymer, and monomer vs polymer). Bivariate relations between different biomarkers and between biomarkers and exposure variables were assessed by computing Spearman correlation coefficients to test whether larger values of one variable tended to be paired with larger values of the other variable, regardless of the functional form of the relation. Unless otherwise noted, correlation results are presented as Spearman correlation coefficients (r) with P values. Finally, multiple regression was used to examine the effects of exposure, as quantified by average BD exposure (or average BD exposure 70–50 days before blood sampling), on various parameters relevant for the *HPRT* assays, such as MF or VF, after adjusting for age, smoking status, CE, LI, or any of several cell viability characteristics and numbers of clonable or evaluatable cells available for the *HPRT* assays. For these parametric analyses, the natural logarithms for BD exposure and MF or VF were used.

MFs and CEs were also analyzed with statistical procedures described by Thomas and associates (1999). A novel aspect of these procedures was that the authors took into consideration the fact that the number of clonable cells per subject often fluctuates considerably. This can result in marked intersubject differences in the accuracy of MFs. The methods described account for such differences by applying *weighted* linear regression so that one can down-weight observations when the calculated outcome is known less precisely. Results obtained with the procedures of Thomas and associates were in complete agreement with those of our conventional statistical analyses and are therefore not reported separately.

Considerable intersubject differences in numbers of clonable or evaluatable cells were noted not only by Thomas and associates (1999) and in our cloning assay but also in the autoradiographic assay (see the later section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Results of the Autoradiographic Assay / Quality of the Lymphocyte Samples). For the autoradiographic assays, any assay that had less than 400,000 ECs (the equivalent of clonable cells in the cloning assay) were excluded from the statistical analysis. For comparative purposes, some of the statistical procedures described above were also applied to a database from which all samples with less than 400,000 clonable cells were excluded.

RESULTS OF THE CLONING ASSAY

MFs

MF information was obtained for 78 (95%) of the 82 subjects analyzed. The assay was unsuccessful for four subjects: one control subject (4%), one monomer production worker (4.3%), and two polymerization workers (5.8%). Of the 78 subjects analyzed, three in the monomer group yielded an MF of 0. For each exposure group, Table 24 contains information on MF: mean ± SD, median with range, and lower and upper (25% and 75%) quartiles. The group mean MFs for the control, monomer, and polymer groups were 13.01×10^{-6}, 9.24×10^{-6}, and 18.83×10^{-6}, respectively. The same information is presented as a box plot in Figure 19 and as a scatter plot for each subject in Figure 20.

In Figure 19, note that the polymer group had one subject (number 137) with a relatively high MF value of 83.55×10^{-6}. Five of this subject's mutant lymphocytes could be analyzed at the molecular level by means of reverse transcriptase–PCR (RT-PCR), or multiplex-PCR, or both. In two mutants, the mutations were identical and involved a deletion of bases 51–76 in exon 2. The other three mutations were: (1) 409 A→G in exon 6, (2) deletion 439–479 plus a base change A→C at 431, and (3) IVS8–3T→G in exon 9 ("IVS" is intervening sequence, or "intron"). Thus, it seemed that the high MF in subject 137 was not due to a single mutational event with subsequent clonal expansion of the mutated cell. For the statistical analysis of MF data, therefore, the outlier data were not corrected for clonality. Excluding the outlier data would have resulted in lowering the MF mean (± SD) for the polymer group from 18.83 (± 17.4) $\times 10^{-6}$ to 16.75 (± 13.0) $\times 10^{-6}$. In Figure 19, however, the outlier value is presented as a separate dot.

Three monomer production workers in the database had MF values equal to zero (subjects 104, 140, and 178). Because the statistical analysis of MF data was predominantly performed on log-transformed MFs, these subjects were automatically excluded from the database because the log of zero does not exist. In Table 24, however, the three subjects with MF = 0 were included for calculating the mean ± SD, median (and range), and quartiles of MFs. After excluding the three subjects with MF = 0, the mean (± SD) MF for the monomer group increased from 9.24 (± 6.24) $\times 10^{-6}$ to 10.70 (± 5.38) $\times 10^{-6}$.

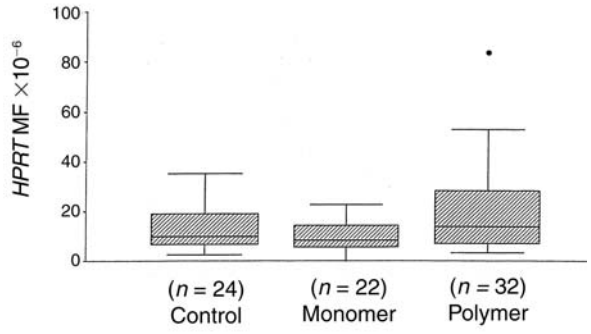

Figure 19. Box plots of *HPRT* MFs for control, monomer, and polymer groups. The *n* values have been adjusted to accommodate (a) one monomer production worker's blood not shipped from Prague to Leiden and (b) four samples lost to unsuccessful cloning: 1 administrative control subject, 1 monomer production worker, and 2 polymerization workers. Data for the three monomer production workers with MF = 0 were included to calculate the mean, median, and quartiles. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. Error bars are the highest and lowest values within 1.5 box-lengths from each percentile. The line within each box marks the position of the median value. One polymerization worker had an MF of 83.55×10^{-6}, which was outside the error bars; this value is indicated with a dot above the box plot.

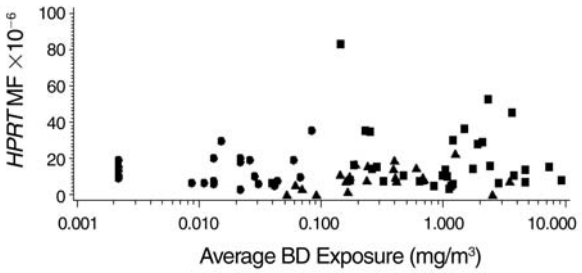

Figure 20. Log-linear scatter plot illustrating the absence of an association between MF and average BD exposure. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

Differences in MFs Among the Exposure Groups

Because the distribution of MFs was skewed, the nonparametric Kruskal-Wallis test was used to test for differences among the three exposure groups. The analysis did not show statistically significant differences ($P = 0.13$).

So that analyses of MF data from the cloning assay could be compared with analyses of VF data collected with the *HPRT* autoradiographic assay, the above analyses were repeated excluding 20 subjects with less than 400,000 clonable cells (the equivalent to ECs in the autoradiographic assay). After excluding these subjects (3 administrative control, 6 monomer production, and 11 polymerization workers), differences in MFs among the three exposure groups became smaller; mean MFs for control, monomer, and polymer groups were then 12.43×10^{-6} ($n = 21$), 10.84×10^{-6} ($n = 16$), and 12.77×10^{-6} ($n = 21$), respectively (see MF for All Subjects, mean ± SD, in Table 24 for comparison values).

Association Between MF and Exposure to BD

Figure 20 is a log-linear scatter plot illustrating the relation between MF and log BD exposure (in mg/m³). Data for subjects of the three exposure groups are presented with different symbols. Average BD exposure was calculated in two ways: using all exposure measurements over the entire assessment period and using only those measurements made 70 to 50 days before blood sampling. The correlations presented below are based on the overall average exposure, but very similar results were obtained using the exposure interval 70 to 50 days before blood sampling.

The association between MF and overall average BD exposure was assessed by computing Spearman rank correlation coefficients (r). The overall association for all three exposure groups combined was not significant ($r = 0.110$; $P = 0.350$). When only results for the monomer and polymer groups were combined, the association was again not significant ($r = 0.140$; $P = 0.324$). Finally, the correlations for the monomer and polymer groups considered separately also were not significant. In conclusion, we found no significant association between MF and BD exposure.

As an alternative statistical approach for the analysis of the exposure data, linear regression lines were estimated for the association between log-transformed MFs and log BD exposure. This was done for the monomer and polymer groups with and without correction of ln MF for CE. For both exposure groups we found no significant association between MF or ln MF and BD exposure.

Effect of Smoking on MFs

After excluding the three monomer production workers with MF = 0 and the one control subject with unknown current smoking status, the remaining subjects were divided into subgroups of 34 current smokers and 40 current nonsmokers. Mean MFs (± SDs) for these subgroups were $13.95 (\pm 7.96) \times 10^{-6}$ for smokers and $15.93 (\pm 16.09) \times 10^{-6}$ for nonsmokers. Using two-way ANOVA on ln MF, we found no significant effect of smoking ($P = 0.385$) and no significant exposure-group–by–smoking interaction ($P = 0.107$); this indicates that the effect of smoking in each of the three exposure groups did not differ significantly. MFs for smokers and nonsmokers in each of the three exposure groups are presented in Table 24. MFs correlated with cumulative cigarette consumption computed as the number of packs of cigarettes smoked per day times the number of

years smoked ($r = 0.325$; $P = 0.005$). (More detailed information on number of packs and years smoked is provided in the earlier section Workers' Exposure Groups, Personal Characteristics, and Metabolic Genotypes / Personal Characteristics / Results: Characteristics and Coexposures.)

Effect of Age on MFs

Because it has frequently been reported that MFs increase with age (Tates et al 1991b; Branda et al 1993; Finette et al 1994), the age distributions in the exposure groups were examined. Mean age ± SD was 38 ± 12 ($n = 24$) for the control group, 42 ± 12 ($n = 19$) for the monomer group (excluding the three subjects whose MF = 0), and 39 ± 12 ($n = 32$) for the polymer group. Age differences among the exposure groups were not statistically significant. By means of a multiple regression analysis for the total database minus the three monomer production workers with MF = 0, we analyzed the relation between ln MF and age. The equation for this relation was calculated to be: $\ln MF = 1.889 + 0.014 \times age$, which indicated that MFs increased significantly with age ($P = 0.041$).

Because an inverse relation between MF and CE has often been observed, the above type of equation was also calculated by taking the effect of CE on MF into account with the following equation: $\ln MF = 2.281 - 0.028 \times CE + 0.015 \times age$. This demonstrated that MFs decreased significantly with increasing CE ($P = 0.025$) and that MFs increased significantly with age ($P = 0.005$). The values of the coefficients for the spontaneous MF, CE, and age were quite similar to values described in other studies on human populations (Tates et al 1991b; Branda et al 1993; Finette et al 1994), which indicates that this adult population in the workforce studied seemed normal and did not remarkably differ from populations studied elsewhere in Europe or the United States (see also the later section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Discussion).

Effect of Genotype on MFs

Evidence is accumulating that mutation induction in human subjects is not only dependent on exposure to mutagens but also on the genotype of exposed subjects. Consequently, MFs can be different in individuals who received the same mutagen exposure but who differed in their genotypes. Molecular techniques have become available to identify genetic polymorphisms in exposed study populations. In the present study, all subjects were genotyped for inherited polymorphisms of the *CYP* isoform *CYP2E1*, for *EH*, for the *GSTM1* and *GSTT1* isoforms, and for *ADH2* and *ADH3*. For this investigation, we used genomic DNA isolated from expanded *HPRT*$^+$, or *HPRT*$^-$, or both clones collected during the study. When the inventory of genotypes was completed, it became possible to correlate individual genotypes or groups of genotypes with MFs for individual subjects or groups of subjects. We found no associations between genotype and MF values.

Correlations of MFs with Urinary Metabolites or Hb Adducts

We found no significant correlations between urinary metabolites or Hb adducts and MF values (see the later section Correlations Among Biomarkers).

CEs

Nonselection CEs (−TG) were measured to obtain information on the capacity of T cells to grow in vitro in the cloning assay and, more importantly, to calculate MFs. Numerous previous studies have shown an inverse relation between nonselection CEs and MF; in the current study, we also observed a significant negative correlation ($r = -0.240$; $P = 0.038$). Nonselection CEs for individual subjects were measured by seeding microtiter plates with three or six cells per well. This resulted in two CE values per subject that served as internal QC standards for each other and that generally corresponded very well. The average of the two CE values was used to calculate MFs for the individual subject, as well as the mean CE, median CE, and range of CEs for each exposure group (see Table 24). CE data from all subjects were used regardless of whether mutant selection was successful or whether MF = 0. Scatter plots of average CE values for all subjects versus BD exposure are provided in Figure 21.

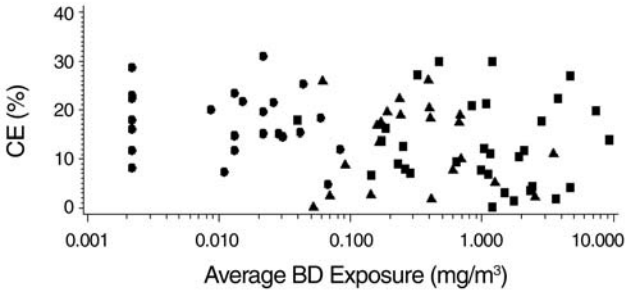

Figure 21. Log-linear scatter plot illustrating the association between CE and average overall BD exposure. CEs decreased with increasing BD exposure. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

Differences in CEs Among the Exposure Groups

Exposure group means for nonselection CEs were 17.5% for the control group, 13.4% for the monomer group, and 12.8% for the polymer group. The difference in percentages among the exposure groups was of borderline statistical significance when analyzed with the Kruskal-Wallis test ($P = 0.054$).

Using two-way ANOVA, we tested for an association between CE and smoking and found no significant effects for either smoking on CE ($P = 0.358$) or for the smoking–by–exposure-group interaction ($P = 0.909$).

Association Between CE and Exposure to BD

Figure 21 is a log-linear scatter plot illustrating the association between CE and BD exposure for all subjects. Using the Spearman test, we found significant negative associations between CE and average overall BD exposure ($r = -0.272$; $P = 0.016$) and between CE and average exposure during the exposure interval 70 to 50 days before blood sampling ($r = -0.356$; $P = 0.002$). This result implies that CE decreases with increasing BD exposure. When control subjects were excluded from the database, the associations were no longer significant (overall exposure: $r = -0.079$, $P = 0.570$; exposure during the interval 70–50 days before blood sampling: $r = -0.154$, $P = 0.297$). Thus, when the statistical analysis was restricted to exposed subjects, we found no evidence for a decrease of CE with increasing BD exposure.

Numbers of Clonable Cells

By using nonselection CE values (−TG) for each subject plus the total number of wells with 20,000 cells in selection plates (+TG; plates containing medium with the TG selection agent), it was possible to calculate the number of clonable cells per subject. This number fluctuated between 33,000 and 5,040,000 cells. Exposure group means (± SD) were 1.12 (± 0.94) $\times 10^6$ for the control group, 1.59 (± 1.37) $\times 10^6$ for the monomer group, and 1.01 (± 1.06) $\times 10^6$ for the polymer group (see Table 24). Analyzing these data with the Kruskal-Wallis test did not show significant differences among the three exposure groups ($P = 0.232$).

Numbers of Living Cells

For each subject, we measured the number of living cells after thawing and at the end of the preincubation period (Table 24). Unlike the results for clonable cells described in the preceding paragraph, we noted significant differences (by the Kruskal-Wallis test) among exposure groups in number of cells alive after thawing ($P = 0.003$) and after preincubation ($P = 0.003$). Monomer production workers had significantly higher numbers of viable cells than administrative control subjects or polymerization workers. The latter two groups did not differ from each other. Differences in cell numbers among exposure groups were not related to BD exposure (cells alive after thawing: $r = 0.017$, $P = 0.880$; cells alive after preincubation: $r = 0.074$, $P = 0.508$).

In addition to cell numbers after thawing and preincubation, we calculated cell recovery (Table 24). Each exposure group had a small number of subjects with cell recoveries exceeding 100% (control 4%; monomer 4%; polymer 17%). A recovery greater than 100% may reflect occasional inaccuracies in cell counting procedures or an increase in cell numbers during preincubation. In the latter case a recovery greater than 100% might mean that some subjects had an infectious disease resulting in active lymphocyte proliferation at the time of blood sampling. The difference among exposure groups was not significant and cell recovery was not associated with BD exposure ($r = 0.043$, $P = 0.710$).

RESULTS OF THE AUTORADIOGRAPHIC ASSAY

Quality of the Lymphocyte Samples

The cryopreserved cells were in transit from Prague, Czech Republic, to Galveston TX for 8 days in dry shippers with liquid nitrogen. Upon receipt, the vials containing lymphocytes appeared to be frozen hard and were rapidly transferred to a Dewar flask filled with liquid nitrogen. The plasma, which was stored in a separate 15-mL tube for each subject, had been shipped in a separate dry shipper. Samples at the top of the shipper were partially thawed, whereas samples in the lower part of the container were still frozen hard. Partial thawing should not have appreciably altered the effectiveness of the plasma as a culture medium supplement. The plasma samples were rapidly transferred to a freezer at −20°C.

When the lymphocytes were cultured to determine the *HPRT* VF, we noted that a significant percentage of samples produced cultures with a very low LI, or low numbers of cells recovered after culturing, or both. We decided that a criterion should be established for acceptable sample quality based on the number of ECs. To do this, the 95% CI for an *HPRT* VF ($\times 10^{-6}$) based on the observation of zero labeled (variant) cells from +TG-selection cultures was calculated for different numbers of ECs (number of cells recovered from the +TG cultures multiplied by the LI). The result of the calculation is shown in Figure 22. When the number of ECs was low, the CI was very wide, exceeding the range of values of VF for the entire data set. At high numbers of ECs, the CI became very small. We

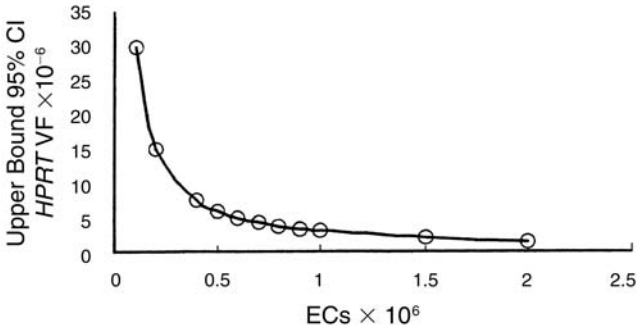

Figure 22. Calculated upper bound of the 95% CI (*HPRT* VF × 10^{-6}) for +TG cultures with zero labeled (variant) cells at different numbers of ECs.

selected the criterion on the basis of a compromise between having an acceptably small CI and retaining an adequate number of samples for analysis. We inspected the curve in Figure 22, selected different values of ECs, tested each one as a possible criterion, and determined the number of samples that would be available for analysis if that value of EC were to be selected as the criterion. On the basis of these tests, we decided to set the criterion at 0.4×10^6 ECs. At that criterion value, the upper bound of the CI is an *HPRT* VF of about 7×10^{-6}, a value in the range of samples from workers in high-exposure areas in our previously published studies of occupational exposure to BD (Ward et al 1994, 1996b, 2001; Ammenheuser et al 2001).

This criterion was applied in compiling a list of the samples that would be included in the final data set before breaking the code. When the code was broken, the numbers of samples in each of the three exposure groups turned out to be as shown in Table 25. The percentages of satisfactory cultures in the control, monomer, and polymer groups were 79, 71, and 52, respectively (the control group value also excludes one sample due to uncertain current smoking status). We also excluded five samples because, when assayed, they produced no labeled (variant) cells (VF = 0; Table 25). Thus the final number of samples that could be adequately analyzed was 48 (58% of the samples received). Of the 27 internal QC samples, 25 met the criterion of more than 0.4×10^6 ECs per culture. The mean ($\pm$ SD) number of ECs for the 25 QC samples was 1.85 ($\pm$ 0.93) $\times 10^6$. The mean number of ECs for all of the Prague samples was 0.94 ($\pm$ 1.00) $\times 10^6$.

Results for VF by Exposure Group

Of the total of 82 samples received, the results from 48 samples were analyzed. The results for the samples by exposure group are summarized in Table 26. The mean *HPRT* VF for the control group was significantly greater than the two groups exposed to BD ($P < 0.05$).

When broken down by exposure group and smoking, we saw little effect of smoking on the *HPRT* VF in the control group, an increased VF in the monomer group (3.84×10^{-6} for nonsmokers vs 7.21×10^{-6} for smokers), and a decreased VF in the polymer group (8.77×10^{-6} vs 4.48×10^{-6}) (Table 26 and Figure 23). For the nonsmokers, the average BD exposures in the monomer and polymer groups (Table 26) were consistent with the trend of the *HPRT* VF, but we found no positive correlation between individual exposures and *HPRT* VF (Figure 24).

Table 25. Suitability of Samples to be Included in Statistical Analyses of *HPRT* VF

	Control	Monomer	Polymer	Total	Internal QC Samples[a]
Samples received	25	24	33[b]	82[b]	27
Sample excluded due to uncertain smoking status	24	NA[c]	NA	81	NA
Criterion of 0.4×10^6 ECs					
Satisfactory samples (*n*)	19	17	17	53	25
Satisfactory samples (% of total available)	79	71	52	65	93
Unsatisfactory samples (*n*)	5	7	16	28	2
Unsatisfactory samples (% of total available)	21	29	48	35	7
Samples eliminated because VF = 0	2	1	2	5	1
Total samples available for statistical analyses	17	16	15	48	24

[a] Samples from subjects in a study being conducted in a Texas SBR plant and from unexposed Texas control subjects.

[b] The sample for one polymerization worker was not shipped from Prague to Galveston for analysis; therefore, for the total subjects, $n = 82$ rather than 83, and for the polymer group, $n = 33$ rather than 34.

[c] NA = not applicable.

Table 26. Summary of Autoradiographic Assay Results for Nonsmokers and Smokers

Parameter	Control[a]	Monomer	Polymer[b]
Nonsmokers			
Cells alive ($\times 10^6$) after thawing			
Mean ± SD	13.47 ± 4.60	21.36 ± 15.55	17.72 ± 8.74
Median (range)	12 (8–28)	16 (3–55)	14.5 (3–38)
n (= 44 total)	15[a]	11	18[b]
Cells recovered ($\times 10^6$) after culture (LI + TG flasks)			
Mean ± SD	4.35 ± 2.56	8.75 ± 5.33	6.09 ± 3.97
Median (range)	3.623 (2.366–12.985)	7.284 (1.312–18.951)	5.034 (1.175–18.493)
n (= 44 total)	15[a]	11	18[b]
ECs ($\times 10^6$)			
Mean ± SD	0.565 ± 0.200	1.778 ± 1.087	1.695 ± 1.059
Median (range)	0.482 (0.403–0.972)	1.432 (0.593–3.751)	1.298 (0.839–3.866)
n (= 23 total)[c]	9	7	7
LI			
Mean ± SD	0.244 ± 0.089	0.251 ± 0.094	0.274 ± 0.077
Median (range)	0.240 (0.08–0.362)	0.249 (0.155–0.400)	0.272 (0.159–0.373)
n (= 23 total)[c]	9	7	7
BD exposure (mg/m^3)			
Mean ± SD	0.017 ± 0.014	0.652 ± 1.276	2.501 ± 3.394
Median (range)	0.015 (0.002–0.042)	0.665 (0.062–3.516)	1.154 (0.040–9.244)
n (= 23 total)[c]	9	7	7
VF ($\times 10^{-6}$)			
Mean ± SD	11.29 ± 8.16	3.84 ± 2.87	8.77 ± 5.64
Median (range)	7.04 (2.48–23.66)	3.70 (0.53–9.33)	5.40 (2.59–19.29)
n (= 23 total)[c]	9	7	7
Smokers			
Cells alive ($\times 10^6$) after thawing			
Mean ± SD	20.67 ± 5.57	25.38 ± 8.10	16.67 ± 7.27
Median (range)	20 (13–30)	27 (11–35)	15 (4–28)
n (= 37 total)	9[a]	13	15[b]
Cells recovered ($\times 10^6$) after culture (LI + TG flasks)			
Mean ± SD	6.50 ± 2.42	9.00 ± 3.46	5.58 ± 2.34
Median (range)	5.158 (4.001–10.391)	8.963 (2.870–14.642)	5.888 (0.782–10.0)
n (= 37 total)	9[a]	13	15[b]
ECs ($\times 10^6$)			
Mean ± SD	1.353 ± 0.893	2.229 ± 1.205	0.946 ± 0.579
Median (range)	0.900 (0.495–3.083)	1.747 (0.685–4.380)	0.770 (0.403–2.22)
n (= 25 total)[c]	8	9	8
LI			
Mean ± SD	0.313 ± 0.106	0.308 ± 126	0.235 ± 0.100
Median (range)	0.274 (0.187–0.486)	0.336 (0.058–0.475)	0.214 (0.120–0.43)
n (= 25 total)[c]	8	9	8
BD exposure (mg/m^3)			
Mean ± SD	0.018 ± 0.019	0.654 ± 0.570	2.038 ± 2.448
Median (range)	0.013 (0.002–0.059)	0.411 (0.146–1.861)	1.025 (0.188–7.40)
n (= 25 total)[c]	8	9	8
VF ($\times 10^{-6}$)			
Mean ± SD	10.32 ± 3.69	7.21 ± 5.48	4.48 ± 2.91
Median (range)	10.84 (5.06–16.54)	4.36 (1.46–15.65)	1.025 (1.18–9.64)
n (= 25 total)[c]	8	9	8
All Subjects for Whom VFs Were Determined			
BD exposure (mg/m^3)			
Mean ± SD	0.018 ± 0.016	0.53 ± 0.903	2.254 ± 2.827
n (= 48 total)[c]	17	16	15
VF ($\times 10^{-6}$)			
Mean ± SD	10.75 ± 6.11[d]	5.73 ± 4.72	6.48 ± 4.77
Median	9.71	3.79	5.40
n (= 48 total)[c]	17	16	15

[a] Data for one administrative control subject were omitted due to unknown smoking status. Therefore, for those analyses involving smokers vs nonsmokers, the total number in the control group was 24 rather than 25.

[b] The blood sample for one polymerization worker was not shipped from Prague to Galveston for analysis; therefore, for total subjects, $n = 82$ rather than 83, and for the polymer group, $n = 33$ rather than 34.

[c] The total number of samples available for statistical analyses was 48 (smokers plus nonsmokers); see Table 25.

[d] Significantly greater than both exposed groups ($P < 0.05$) by Kruskal-Wallis test.

The correlations between individual *HPRT* VF values and urinary metabolites or Hb adducts were all negative (see the later section Correlations Among Biomarkers).

ECs

The numbers of ECs were not equally distributed between the nonsmokers and smokers in the three exposure groups (Table 26). The assays for the 9 nonsmokers in the control group for whom VFs were obtainable produced an average of only 0.565×10^6 ECs. This is only slightly above the minimally acceptable criterion of 0.400×10^6 used in the present study, and is four times lower than the numbers of ECs obtained in our three studies of BD-exposed and control subjects in Texas (Ward et al 1996b, 2001; Ammenheuser et al 2001). The CIs are quite wide for the VFs obtained with the low numbers of ECs in the control nonsmoker group when compared with the CIs of the monomer nonsmoker group (1.778×10^6 ECs) and the polymer nonsmoker group (1.695×10^6 ECs). The other group with a relatively low mean number of ECs was the polymer smoker group. That mean level of ECs (0.946×10^6) was half of the mean number of ECs in the monomer smoker group (Table 26).

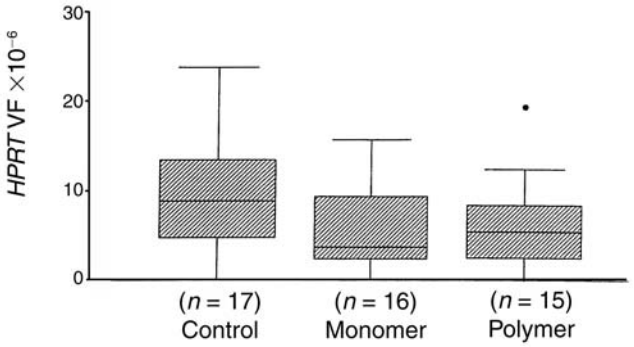

Figure 23. Box plots of *HPRT* VFs for control, monomer, and polymer groups. The *n* values have been adjusted to accommodate sample sizes selected for statistical analysis as described in Table 25. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. Error bars are the highest and lowest values within 1.5 box-lengths from each percentile. The line within each box marks the position of the median value. One polymerization worker had a VF of 19.29×10^{-6}, which was outside the error bars; this value is indicated with a dot above the box plot.

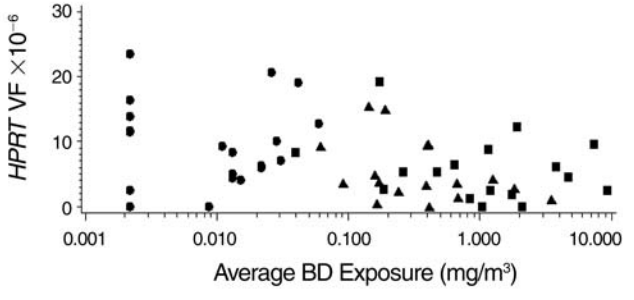

Figure 24. Log-linear scatter plot illustrating the lack of a significant association between VF and average BD exposure. ● = administrative control subjects; ▲ = monomer production workers; and ■ = polymerization workers.

Consistency of Slide Scoring

Scoring slides generated in the autoradiographic assay has the potential for errors because of the somewhat subjective judgments made in identifying labeled cells under the microscope. Differences in the visual acuity or judgment of the observers can produce different results. This was controlled in two ways. First, the blind coding of the slides assured that the scorers had no knowledge of the origin of the slides at the time they were evaluated. Second, the slides were scored in Galveston and again independently in Burlington. Before scoring the slides from the current study, the two laboratories examined a set of slides generated from other studies at the University of Texas Medical Branch and compared the results until we had jointly agreed on criteria for identifying labeled variant cells. Then the cells from Prague were cultured and the slides were scored by both laboratories. The comparison of results from the two laboratories is shown in Figure 25. Although the laboratories did not agree well on a few samples, the overall correlation was excellent ($r = 0.86$).

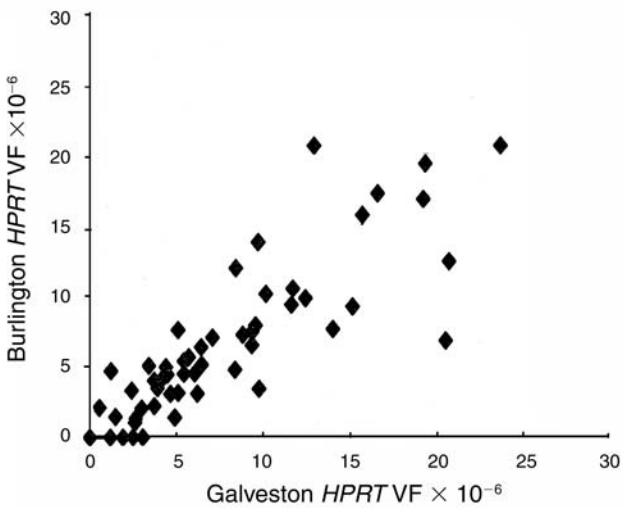

Figure 25. Comparison of *HPRT* VFs determined by scoring slides at Galveston and Burlington. Each data point represents one subject; the five subjects with VF = 0 were included in this comparison.

COMPARISON OF AUTORADIOGRAPHIC AND CLONING ASSAYS

Using Spearman correlation, we calculated that the VFs determined in Galveston were unrelated to MFs determined in Leiden on aliquots from the same blood samples ($r = 0.175$; $P = 0.239$). This correlation did not improve substantially when the analysis was restricted to the 44 samples from subjects with at least 400,000 evaluatable or clonable cells for both assays ($r = 0.202$; $P = 0.187$). The correlation between VFs and MFs, or rather the absence of a correlation between the two parameters, is shown in Figure 26.

The number of ECs per subject in Galveston was significantly correlated with the number of clonable cells in Leiden for aliquots of the same blood samples ($r = 0.601$; $P = 0.001$). The correlation was also statistically significant for each of the three exposure groups (control: $r = 0.657$, $P = 0.001$; monomer: $r = 0.639$, $P = 0.001$; polymer: $r = 0.608$, $P = 0.001$). However, the samples with less than 400,000 evaluatable or clonable cells determined at the two study sites did not completely coincide.

Spearman correlation was also applied to test whether LI values for samples analyzed in Galveston were correlated with cloning efficiencies for the corresponding blood samples analyzed in Leiden. For the total database the correlation was statistically significant ($r = 0.420$; $P = 0.001$).

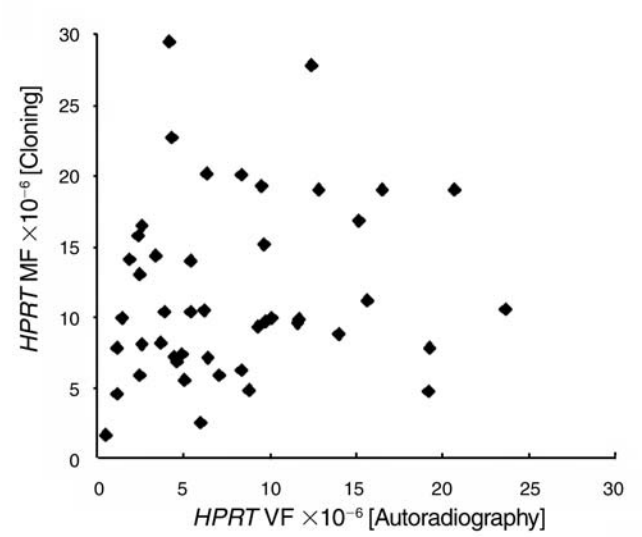

Figure 26. Scatter plot illustrating the absence of a correlation between MFs measured with the cloning assay in Leiden and VFs determined with the autoradiographic assay in Galveston on the same blood samples. Samples in which either assay produced a 0 value have been eliminated from the graph. ● = administrative control subjects; ▲ = monomer production workers; and ■ = polymerization workers.

DISCUSSION

The Cloning Assay

By means of the cloning assay, it was not possible to detect statistically significant differences among the MFs of control, monomer, and polymer exposure groups. The mean MF for the monomer group, after excluding three subjects with MF = 0, was 18% lower than that for the control group. In contrast, the mean MF for the polymer group (outlier included) was 45% higher than the control group.

Because of this increase of the mean MF for the polymer group, one must ask whether it would have been statistically significant if a larger group of polymerization workers had been studied. In this connection, Robinson and colleagues (1994) calculated the number of subjects per group required to detect a given difference in MFs between two exposure groups with a 90% chance of $P < 0.05$. Under the experimental circumstances of our investigation, and with a table provided by Robinson and colleagues (1994), it can be calculated that around 60 subjects per study group would be required to detect a statistically significant 45% increase (1.46-fold) of the MF for polymerization workers. MFs in the present study were measured for not more than 32 polymerization workers. The same table in the Robinson publication indicates that analyzing data from about 30 subjects would detect a statistically significant 70% increase 90% of the time.

The 32 polymerization workers in the current study comprised about half the work force of that production facility. The other half worked for an independent contractor who chose not to participate in our investigation. The monomer production workers that we studied comprised the entire production staff of that facility. Only a few workers employed by the primary industry refused to participate. In conclusion, this investigation was performed with as many workers as were available; and yet, with the cloning assay (and the autoradiographic assay as well), we did not detect a significant induction of *HPRT* mutations in either of the two groups of exposed workers compared with each other or with the control group. As noted, the number of workers available for the study may have been too small to reliably detect small average group increases in MF as being statistically significant. However, we also did not find any positive associations between MFs and BD exposures for individual workers.

This negative finding with the cloning assay is consistent with results of two other cloning assay studies, one of which was conducted in our own laboratory (Tates et al 1996; Hayes et al 2000). In our 1993/1994 study, blood samples were drawn from workers in the same monomer production unit that was studied in the current investigation

(Tates et al 1996). In 1994 the mean exposure level was 3.89 mg/m^3 ([1.76 ppm] range: 0.03–43.69 mg/m^3 [0.01–19.77 ppm]). The mean BD level for monomer production workers was higher than that measured with much greater accuracy in the current study (mean: 0.64 mg/m^3 [0.29 ppm]; range: 0.053–3.516 mg/m^3 [0.02–1.59 ppm]) and also higher than the mean for the polymerization workers in the present study (1.79 mg/m^3 [0.81 ppm]; range: 0.04–9.24 mg/m^3 [0.02–4.18 ppm]).

It is remarkable that mean MFs for monomer production workers in the past and present studies at the same facility (past: 10.10 [± 10.32] × 10^{-6}, n = 24; present: 9.24 [± 6.24] × 10^{-6}, n = 22) were lower than mean MFs for the control group (past: 13.93 [± 9.04] × 10^{-6}, n = 26; present: 13.01 [± 8.10] × 10^{-6}, n = 24). Evidently, these results are in good agreement despite the fact that several differences exist between the two studies. First, the control group for the present study consisted of volunteers from a large pool of male administrative workers outside the monomer production unit, whereas the control group from the previous study consisted of male workers from a heat production unit of the monomer plant. Second, cloning assays for the past and present studies were performed in the same laboratory by practically the same technical staff, but the protocols used were different in two aspects: (1) type of lymphocyte growth factor (past: LAK supernatant as the source of IL-2; present: recombinant IL-2); and (2) procedures used for priming/stimulating lymphocytes (past: preincubation for 44 hours in the presence of LAK supernatant followed by counting of stimulated/primed lymphocytes; present: preincubation for 24 hours without IL-2 source followed by counting of live cells regardless of whether or not they were stimulated).

The main reason for modifying the cloning assay in the present study was that we wanted to adapt as much as possible to the Uniform Operating Protocol, which had been developed by a group of European laboratories (Hou et al 1999) with the objective of rigidly standardizing the cloning assay protocols so that results from different laboratories and countries could more reliably be compared and perhaps even pooled. In terms of the values for CEs in the absence of TG selection, the Uniform Operating Protocol was not necessarily better or worse than the protocol we had used previously for other biomonitoring studies, including our earlier BD study (Tates et al 1996). In comparing the two studies, we found little or no difference between mean CEs for the control group in the past study (15.48%, range: 2.7–39.4; see Table 3 in Tates et al 1996) and the control group in the present study (17.51%, range: 4.8–31.1). For the monomer group, mean CEs for the past and present studies were, respectively, 19.90% (range 8.2–33.8) and 13.43% (range 0.4–26.5).

On the basis of these comparisons between MFs and CEs for the control and monomer groups in the past and present studies, we conclude that the differences in results are negligible despite differences in the origin of the control populations and differences in protocols used for the cloning assay.

Apart from the consistency of results between the two BD studies performed in our laboratories over a time span of about 5 years, we have several "sentinel findings" in the current study to suggest that the cloning assay was performing satisfactorily. First, in agreement with other published findings (Cole et al 1990; Tates et al 1991b; Branda et al 1993; Finette et al 1994), we found that MFs decreased significantly with increasing CEs, whereas MFs increased significantly with age (for details see the appropriate earlier subsections under Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Results of the Cloning Assay). The values of the coefficients that we found for spontaneous MFs, CEs, and age were quite similar to the ones calculated in other studies on human populations; this indicates that the current study population seemed normal (in so far as the cloning assay results are concerned) and did not remarkably differ from adult populations studied elsewhere in Europe and the United States. Second, the present study indicated a positive correlation between MFs and cumulative smoking history (see the earlier section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Results of the Cloning Assay). This finding also provides confidence that the cloning assay was functioning properly. Finally, during the entire cloning assay portion of the study, an internal QC standard was analyzed concurrently with each set of six Czech samples. Throughout the study, CEs for the QC standards fluctuated very little, which indicates that the cloning assay was performed consistently even though fresh medium was prepared for each set of six Czech samples. Furthermore, not only was the MF for the monomer group lower than that for the control group, but in the parallel autoradiographic study, the VF for monomer group was also lower than that for the control group.

The absence of statistically significant mutagenic effects of BD exposure in our past and present Czech study populations is consistent with observations of Hayes and coworkers (2000) who also used a cloning assay and could not demonstrate induction of *HPRT* mutations in lymphocytes from BD-exposed workers in China. The investigators of the study in China did not detect a significant increase in the frequency of glycophorin A mutants in erythrocytes of the same exposed workers when compared

with control subjects, nor did they find BD-associated genetic effects at the chromosomal level (such as SCEs or aneuploidy). As in our own study, *HPRT* MFs did not correlate with any of the other parameters tested in the study. Median air levels of BD for dimethylformamide and polymerization process analysts were 2.21 and 7.74 mg/m^3 (1.0 and 3.5 ppm), respectively. These exposure levels are higher than the median levels of BD for the monomer and polymer groups in our present investigation. Ma and associates (2000) reported that a BD-exposed worker group in an SBR plant in Texas USA had a significantly higher MF than the nonexposed control group ($P < 0.05$). Workers evaluated for MF with the cloning assay were exposed to BD at a median level of 1.90 mg/m^3 (0.86 ppm). The median BD exposure level in that study was somewhat higher than the median level of 0.599 mg/m^3 (0.285 ppm) for the polymer group in the current study. The mean CEs of the samples from the Texas SBR workers were also considerably higher than those for either of the Czech studies (Texas study: 32.1% for the control group and 28.7% for the polymer group [Ma et al 2000]; 1994 Czech study: 14.0% for the control group and 19.9% for the polymer group [Tates et al 1996]; current Czech study: 17.5% for the control group and 12.8% for the polymer group). We cannot explain the discrepancies among the China study, the two Czech studies, and the Texas study.

As discussed earlier, BD exposure has been found to be mutagenic in mice and rats. Several researchers have investigated the molecular nature of *HPRT* mutations induced by BD and its metabolites in lymphocytes of treated mice and rats (Cochrane et al 1994b; Recio et al 1998; Meng et al 2000). Such studies provide insight into the metabolites, adducts, and DNA lesions that contribute to mutagenesis in the two species. When we carried out our own investigations with BD-exposed workers in the Czech Republic, it was not known a priori whether MFs for monomer production and polymerization workers would be different from those for control subjects and, if not, whether it would make sense to perform an extensive molecular analysis of mutant clones. In the absence of such prior knowledge, we therefore decided at the onset of the study to select, expand, and freeze a large number of mutants from occupationally exposed workers and control subjects (in total, 446 mutant clones and 343 wild-type clones were available for various kinds of molecular analysis). Because we did not find significant differences in the MFs of the three exposure groups, we did not expect to find striking differences in the mutational spectra of the three exposure groups. However, as demonstrated by the studies of Hüttner and Holzapfel (1996), the absence of differences in MFs among exposure groups does not exclude the possibility of finding differences in mutational spectra between exposed and control subjects. Those authors used multiplex-PCR to measure the frequency of large deletions among *HPRT* mutations isolated from lymphocytes of vinyl chloride–exposed and matched control subjects. MFs for the two groups were not different; nevertheless, large deletions were found in 21% of the mutants from vinyl chloride–exposed individuals, whereas only 11% of the mutants from the control subjects carried a large deletion. In the same study, investigators found a slight, but statistically significant, difference in the frequency of chromosomal aberrations in vinyl chloride–exposed workers (1.47%) versus control subjects (1.07%). Our present study of Czech workers indicated no difference in the frequency of aberrations among the three exposure groups; in one of our earlier Czech studies, however, we did find a small but significant difference between frequencies of aberrations in BD-exposed monomer production workers when compared with the control subjects, although an earlier study had found no such increase (Tates et al 1996).

In view of these results, a small possibility existed that differences in mutational spectra among the three exposure groups would be found. That molecular analysis of mutant cells from the current subjects would be worthwhile was also suggested by the results of an investigation of BD-exposed workers in the USA (Ma et al 2000). In that study, the spectra of mutations in the *HPRT* gene in lymphocytes from nonsmoking subjects who worked in areas of relatively high exposure to BD (TWA about 3.5 ppm or 7.7 mg/m^3) were compared with the mutational spectra in nonsmoking control subjects. In the BD-exposed subjects, investigators detected a significant increase ($P < 0.05$) in the proportion of mutations involving partial gene deletions and -1 frameshift mutations. A molecular analysis of mutant cells from the subjects in the current study was, therefore, undertaken. We saw no differences between the mutational spectra for control and exposed subjects. The detailed results are presented in the later section Biomarkers of Effect: *HPRT* Mutational Spectra.

The Autoradiographic Assay

Sample Quality The autoradiographic *HPRT* mutant lymphocyte assay requires a relatively large number of lymphocytes in good condition. The assay is based on the detection of rare TG-resistant cells in a background of normal TG-sensitive cells that do not incorporate [^{3}H]TdR. In individuals who do not smoke and have no other risk factors, typical VFs are in the range of 1 to 4 mutants per million ECs (Ammenheuser et al 1997b). If the LI were 12%, the number of ECs in a 30-mL blood sample after lymphocyte

isolation, freezing, thawing, and 42 hours of culture would be about a million cells. At a VF of 2×10^{-6}, a total of only two labeled cells would be present, divided between two slides. However, the VF would appear to be half of that, only 1×10^{-6}, if only one labeled cell was not detected when the slides were scored. The success of the assay as a biomarker is, therefore, highly dependent on the number and quality of the cells available.

This was a major challenge in this study. The lymphocytes collected from the study participants were shared among three laboratories for the cloning and autoradiographic *HPRT* assays and for cytogenetic analysis, which limited the supply available for any one assay. In addition, maintaining the quality of the samples was a concern. The lymphocytes required cryopreservation, both to preserve them for shipping and to minimize the detection of phenocopies in the autoradiographic assay (Albertini et al 1982; Ammenheuser et al 1988). Shipping over the long distance between the Czech Republic and Texas posed additional risks to sample quality because they were about eight days in transit. Although the lymphocytes appeared to be solidly frozen, it became apparent during the assay of the samples that many had poor recovery when thawed and assayed, or low LIs resulted in small numbers of ECs, or both. With a low number of ECs it is likely that no mutant cells would be observed, but that the CI around a value of 0 could be larger than the range of VFs that we had expected on the basis of previous experience with the assay. For this reason, we decided to establish a criterion for acceptable sample quality on the basis of the number of ECs before learning the origins of the samples.

In terms of ECs, the quality of the samples was lower than we have experienced in studies with samples collected locally. For example, in a study of 37 workers in an SBR plant in Texas who were evaluated for *HPRT* VF at about the same time as this study (Ward et al 2001), the average number of ECs was 2.04×10^6. Some of these lymphocyte samples and some samples from nonindustrial control subjects ($n = 25$ total) were assayed concurrently with the samples for this study (as internal QC standards), and the average number of ECs was 1.85×10^6. This was considerably higher than 0.94×10^6, the average number of ECs observed for subjects in the present study. The samples that did not meet the criteria for acceptability were not equally distributed among the three exposure groups (Table 25). Of the samples from the polymerization workers, 48% did not meet the criterion; whereas only about 30% of the samples from subjects in the monomer group were not acceptable. In the control group, no samples from the smokers were unacceptable, but 40% of the samples from the nonsmokers were unacceptable. We were unable to identify a reason for the samples from the polymer group and the control nonsmoker group to be of poorer quality than others. We found no discernable pattern of days on which the unsatisfactory samples were collected, nor were BD levels consistently higher or lower in the subjects with unsatisfactory samples.

Relation of* HPRT *VF to BD Exposure The mean *HPRT* VFs for the nonsmokers in the monomer and polymer groups compared with each other in what appeared to be a BD dose–related trend. The mean VF for the control group, however, was higher than that for either of the two groups exposed to BD. Because individual *HPRT* VFs for monomer production and polymerization workers did not correlate with the individual average BD exposure measurements or with the concentration of urinary metabolites or Hb adducts, BD exposure levels could not have been the reason for this difference between means in nonsmokers. Thus, there is no apparent relation between BD exposure at the current levels detected in the Prague workers and the VF in *HPRT* lymphocytes using the autoradiographic assay (Figure 24).

This result is inconsistent with the earlier studies in our laboratory of *HPRT* mutations in subjects living in Texas. The most obvious differences are in the frequencies of *HPRT* variant lymphocytes observed in nonsmokers when our four studies of Texas BD workers (Ward et al 1994, 1996b, 2001; Ammenheuser et al 2001) are compared with the current study. For example, in our 1992 study of workers in a BD monomer plant in Texas (Ward et al 1996b), the average VF of the low-exposure nonsmokers ($n = 8$) was 2.14×10^{-6}; and in our 1998–1999 study of workers in a polymerization plant in Texas (Ward et al 2001), the VF for low-exposure nonsmokers ($n = 13$) was 3.61×10^{-6}. Furthermore, in a study in the United States that compared the *HPRT* VFs for smokers and nonsmokers (Ammenheuser 1997b), we reported a mean VF of 1.74×10^{-6} for 42 nonsmokers with no known exposure to mutagenic agents. Even the heavy smokers in that 1997 study had a mean VF of only 8.09×10^{-6}, which is lower than the VF of 11.29×10^{-6} for control nonsmokers in the current study. Thus, this unusually high control value could have obscured a positive response in the BD exposure groups.

It does not appear likely that the unusual results observed with the autoradiographic assay are the result of errors made in scoring the slides. The repeat scoring of the slides in Burlington was done to control for this possibility. The close agreement between the analyses done at Galveston and Burlington indicates that the scoring was reasonably accurate.

One of the original reasons for selecting BD manufacturing

facilities in the Czech Republic for this study was that the workers were believed to have a relatively high level of exposure to BD, thus providing a good opportunity to observe responses in biomarker assays. As it turned out, this was not the case. Table 27 summarizes the exposures of the workers who were evaluated for *HPRT* MFs in the previous studies described in the introductory material to this section. In all of these studies, a few high values influenced the mean values, so the medians should also be considered for comparisons. As shown in Table 27, the mean and median BD exposures in the polymerization unit in this study are lower than those in the high-exposure areas of the polymerization plant studied in Texas in 1994–1995 and again in 1998. The exposures in the Czech monomer production area in this study are the lowest of any of the facilities studied. The one study in which exposures appeared to be quite high was the study in China, but Hayes and coworkers (2000) have indicated that these elevated values are largely due to brief and intermittent high exposures. The mean *HPRT* VF (6.48×10^{-6}) for the Czech polymer group in this study is similar to the mean high-exposure value (6.5×10^{-6}) in the Texas 1994–1995 study (Ammenheuser et al 2001), and somewhat lower than the average for all of the high-exposure workers (10.3×10^{-6}) observed in the 1998 Texas study (Ward et al 2000). The average *HPRT* VF (5.73×10^{-6}) for the monomer group in this study was somewhat higher than the average in the low-exposure areas in the two Texas studies (2.14×10^{-6} in 1994–1995 and 3.61×10^{-6} in 1998; data not shown). The BD levels for the Texas low-exposure groups were less than 0.55 mg/m^3 (0.25 ppm) in 1994–1995 and 0.145 mg/m^3 (0.07 ppm) in 1998. The mean *HPRT* VFs for the monomer and polymer groups in the current study are consistent with the values in the Texas studies when the BD exposures are the basis for comparison among the different groups of workers. Despite these comparisons of group means and medians, however, in the current study the range of values for individual monomer production and polymerization workers were quite large, with maximal individual mean exposure levels up to 3.5 mg/m^3 for the monomer production workers and 9.2 mg/m^3 for the polymerization workers over the entire exposure assessment period. There was no correlation between an individual's BD exposure and his *HPRT* VF.

The results of the *HPRT* autoradiographic assay for variant lymphocytes, therefore, do not reveal a mutagenic response to BD in the current Czech worker population. Both the monomer and polymer groups have mean *HPRT* VFs that are significantly lower than the control group. In addition, the *HPRT* VFs in the monomer and polymer groups do not correlate with the individual BD exposures measured by either air monitoring or the exposure biomarkers (urinary M2 and M1, and Hb adducts). The exposure levels in the monomer group were sufficiently low that, on the basis of our earlier experience, the sensitivity of the assay may have been near its limits. In the polymer group, the mean and median BD exposure levels were also lower than the levels measured in our studies in Texas. Those studies detected increases in *HPRT* VF that correlated with individual exposures (Ward et al 1996b, 2001; Ammenheuser et al 2001).

The distribution of *HPRT* VFs in the control nonsmoker group in this study included three samples with VFs between 19 and 24 $\times 10^{-6}$ and one with a VF of about 14 $\times 10^{-6}$. These are an order of magnitude higher than the *HPRT* VF values we have seen previously in nonsmoker subjects

Table 27. Comparison of BD Exposures in the Current Study with Earlier BD Biomonitoring Studies

	BD Exposure				
	Mean ± SD		Median		
Study and Group	mg/m^3	ppm	mg/m^3	ppm	References
Texas 1994–1995: high-exposure polymer	7.03 ± 2.72	3.18 ± 1.23	1.15	0.52	Ammenheuser et al 2001
Texas 1998: high-exposure polymer	3.71 ± 6.67	1.68 ± 3.02	0.92	0.41	Ward et al 2001
Czech Republic 1994: monomer	3.89	1.76	0.53	0.24	Tates et al 1996; Šrám et al 1998
China 1994	Not available	—	~ 4.42	~ 2.00	Hayes et al 2000
Czech Republic 1998: polymer	1.79 ± 2.11	0.81 ± 0.95	0.60	0.27	The current study
Czech Republic 1998: monomer	0.64 ± 0.85	0.29 ± 0.38	0.27	0.12	The current study

who have had no known exposure to mutagenic agents (Ammenheuser et al 1997b, 1998). The VFs of these four control subjects were also much higher than the mean VF of 1.64×10^{-6} obtained from concurrent assays of four unexposed Texas subjects. One of the four control subjects with a high VF also had a chronic autoimmune condition, a recent infection, and a vaccination a few weeks before blood sampling. Another two of these control subjects with high VFs reported exposures to potentially mutagenic agents other than BD. Of the five remaining subjects in the group designated as the control nonsmoker group, two subjects reported that they smoked cigars or pipes. Their VFs of 7.04 and 6.02×10^{-6} are values that are similar to the average VF previously reported for light cigarette smokers (< 10/day) (Ammenheuser et al 1997b). The mean VF for the remaining three nonsmokers in the control group was 3.69×10^{-6}, which is close to the VF for the monomer nonsmoker group (3.84×10^{-6}) but lower than the VF for the polymer nonsmoker group (8.77×10^{-6}). The mean VF (10.32×10^{-6}) for the eight smokers in the control group in the current study is only slightly higher than the mean VF previously reported for 18 heavy smokers (8.09×10^{-6}) (Ammenheuser et al 1997b).

In the polymer group, half of the eight smokers were relatively light tobacco users (about 11 cigarettes per day). Their average BD exposure of 1 mg/m^3 (0.45 ppm) was low, and their mean VF of 2.05×10^{-6} was also low, even for light smokers. However, the average number of ECs obtained in the *HPRT* assays of these subjects was only 0.636×10^6, and only one or two labeled (variant) cells were identified for each subject (mean 1.25 variants). This resulted in large CIs for these VFs (see Figure 22). The mean VF of 4.48×10^{-6} for all of the eight subjects identified as smokers in the polymer group in this study is comparable to the mean of 5.22×10^{-6} obtained in light smokers with plasma cotinine values of less than 90 ng/mL (Ammenheuser et al 1997b). (We do have confidence in our assessment of smoking behavior in the Czech workers because of the expected effects seen for *HPRT* mutations determined by the cloning assay and the increase in SCEs and HFCs determined by cytogenetic assays [see the later section Biomarkers of Exposure: Cytogenetic Analysis].) It is also notable that, in comparison with our previous studies, the worker groups with mean *HPRT* VFs that seemed aberrant (the polymer smoker group and the control nonsmoker group) were the two groups of workers that had the lowest number of ECs. The mean ECs of these two groups were 2 to 3 times lower than the means for the other four groups, resulting in much wider CIs for the VFs of these subjects (see Table 26; Figure 22; the earlier section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Results of the Autoradiographic Assay / Quality of the Lymphocyte Samples; and the earlier section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Discussion / The Autoradiographic Assay / Sample Quality).

Factors That Could Affect the Results of* HPRT *Assays It is of obvious value to know what factors may have contributed to the high VF values detected with the autoradiographic assay in the control group. Because this study is considered transitional in nature, the objectives of the study were to determine the suitability of the biomarker assays as much as they were to evaluate exposures. It is important, therefore, to identify factors that can influence the response of biomarker assays.

Several factors might cause an unusual increase in the *HPRT* VF. An obvious possibility is concurrent exposures to mutagens other than the agent that is the focus of the study. Administrative workers were selected as the control population because their exposure to BD and styrene was estimated to be very low compared with the production workers. This was, in fact, confirmed by the exposure assessment; but some of these administrative workers could have been exposed to other, unknown or unreported, mutagenic agents. This seems highly unlikely, however, because the *HPRT* cloning assay produced results that would be expected for adults who have not been exposed to mutagens.

Another possibility might be the stimulation of T cell replication in some subjects by endogenous factors such as autoimmune disease, infections, or recent immunizations. Such factors might increase the observed frequency of mutant cells in three ways. First, chronic T cell stimulation creates a population of proliferating cells that are at greater risk for either spontaneous or induced mutation than are quiescent (G_0) lymphocytes. Studies of patients with rheumatoid arthritis have demonstrated a fivefold increase in the MF in *HPRT* lymphocytes compared with control subjects (Cannons et al 1998). Second, the replication of mutant cells can lead to the formation of a clone of cells derived from a single mutant progenitor. Such sibling clones can be distinguished from independent mutants by analyzing the spectrum of DNA sequences in T cell receptor genes and the *HPRT* gene (Nicklas et al 1986, 1989). Neither of these seem likely, however, because their effect would be expected to be at least as pronounced in the cloning assay as in the autoradiographic assay for *HPRT* mutations. Third, the persistence of *HPRT* variant or mutant lymphocytes is not well understood. In early studies of the mutagenic effects of cytotoxic drugs and radiation therapy, the *HPRT* VF rose dramatically in 14 days and fell to near baseline levels by 1 month after exposure (Ammenheuser et al 1988, 1991).

The persistence of mutants that are induced by chronic noncytotoxic exposures is probably longer, but the duration is not known. If some *HPRT* mutant lymphocytes persist for periods as long as 6 to 10 years, as appears to be the case for Chernobyl (Russia) nuclear power plant workers (Thomas et al 1999), then a more detailed history of past occupational exposure and past exposures to other potentially mutagenic agents would be needed.

The present study had less than twenty acceptable samples in each exposure group. The presence of a few aberrant values could have had a disproportionate effect on the *HPRT* VF, especially in the control group.

Comparison of the Cloning and Autoradiographic Assays

The measure of *HPRT* T cell mutations, an important biomarker of effect, was assessed by two methods: (1) determining *HPRT* MF by the cloning assay, and (2) determining *HPRT* VF autoradiographically. The results of these two methods were not correlated with each other. However, neither method showed an effect of BD exposure.

The autoradiographic assay is a short-term assay employing DNA synthesis detected by autoradiography. It detects early events in the cell cycle but destroys the cells, which cannot then be used to verify mutational changes. Because of this inability to verify changes, mutant cells are identified by phenotype and are termed variants. The number of variants in blood is expressed as a VF, which is the number of variants per million lymphocytes (ECs) in the assay. VFs are expressed $\times 10^{-6}$.

The cloning assay for measuring *HPRT* mutant T lymphocytes in blood requires longer-term cell cultures. Mutant cells are recognized as growing colonies, which provide material to verify the mutational changes. Mutant cells identified in the cloning assay are termed mutants. The number of mutants in blood is expressed as an MF, which is the number of mutants per million ECs in the assay. MFs also are expressed $\times 10^{-6}$.

Although both assays measure the frequencies of PBLs with mutations in the *HPRT* gene, they may not measure the same cell populations. Therefore, they may not produce the same numerical result for the same blood sample. A previously conducted direct comparison of VF and MF values from the same blood samples also failed to find a one-to-one correlation although, in general, the majority of values that were elevated using one assay were also elevated using the other (Albertini et al 1988).

Conclusion

The primary objective of this work was to determine whether *HPRT* mutant lymphocytes (identified and assessed using two common assays, cloning and autoradiography) are useful biomarkers for evaluating low-dose occupational exposure to BD. Both forms of the assay were used to determine *HPRT* MFs and VFs in lymphocytes obtained from workers after an extensive assessment of their exposure. The BD exposure assessment and the measurements of Hb adducts and urinary metabolites documented that administrative control subjects had the lowest (but measurable) BD exposures, workers in the monomer production unit had been exposed to relatively low levels of BD, and workers in the polymerization unit had been exposed to higher levels of BD. Within the monomer and polymer groups, however, exposure levels spanned large ranges.

Neither form of the *HPRT* mutant lymphocyte assay detected a mutagenic effect of exposure in this study. The results of the cloning assay were consistent with a previous study of workers in the same facility (Tates et al 1996) and with a study of workers in China (Hayes et al 2000). The results of the autoradiographic assay differed from previous studies of workers in the United States in two ways. The control group in this study had unusually high VFs compared with previous studies in Texas, including a study of BD-exposed workers conducted concurrently with this one (Ward et al 2001). Also, although the polymerization workers as a group had an average *HPRT* VF that was somewhat greater than that for the monomer production workers as a group, we found no correlation for any individual between his VF and his average BD exposure. In previous studies (Ward et al 1994, 1996b, 2001; Ammenheuser et al 2001), the autoradiographic assay has proven to be sensitive and responsive to mean BD exposures of about 2 to 7 mg/m^3 (1–3 ppm). In the present study, the mean level of BD was only 0.64 mg/m^3 in the monomer group and 1.76 mg/m^3 in the polymer group (both less than 1 ppm), although individual workers' mean exposure levels, which were used to identify correlations, spanned a far greater range.

In order to make the *HPRT* mutant lymphocyte assays more effective as biomarker assessment tools, it will be necessary to develop a better understanding of the influences of lifestyle, health, and other exposures on *HPRT* VF and MF. In addition, the life span of mutant lymphocytes in the peripheral blood must be better characterized in order to understand the effects of previous exposures to mutagens on *HPRT* VF and MF. For whatever reason, however, the present study could not demonstrate that BD, at the exposure levels evaluated, was mutagenic to the exposed workers.

BIOMARKERS OF EFFECT: *HPRT* MUTATIONAL SPECTRA

Numerous in vitro studies have been performed to determine the mutational spectra of various chemicals and other agents. A mutational spectrum is a description of the kinds of mutations produced in a given situation or following a given treatment. Once a spectrum has been determined for a particular agent, the signature mutations can be searched for in animals and humans exposed in vivo as confirmation of genetic changes due to exposure to that agent. Signature mutations can confirm exposure even when the increases in MFs are small. For example, radiation exposure such as radioimmunotherapy caused an increase in *HPRT* deletions (Nicklas et al 1990), whereas increases in mutations at base pair (bp) 617 were seen in workers exposed to ethylene oxide (Vrieling et al 1992).

Although BD itself is not mutagenic, the BDO and BDO_2 derivatives (and BDO-diol to a lesser extent) are mutagenic both in vitro and in vivo in animals (Cochrane and Skopek 1994a; Tates et al 1998; Recio et al 2000; Walker and Meng 2000). Whereas BDO can form covalent adducts with DNA at the N7 position of guanine or the N6 position of adenine, BDO_2 can also form intrastrand and interstrand cross links, which means it has the potential to cause strand breaks and deletions (Lawley and Brookes 1967; K Peltonen, personal communication 1999).

A number of studies both in vitro and in vivo have been performed to determine the spectra of mutations induced by BD and its metabolites (summarized in Table 28). Most have found increases in A•T→T•A transversions for BD in vivo and for BDO or BDO_2 in vitro. Some studies have found increases in G•C→A•T transitions, A•T→G•C transitions, or A•T→C•G transversions as well. The frequencies of frameshift mutations have been variable. BD exposures in vivo (metabolized to BDO_2) or BDO_2 exposures in vitro appear to increase the frequencies of deletion mutations as would be expected for an agent that causes cross links. However, the methods used to analyze the *HPRT* mutants could not detect large deletions.

As presented in the previous section (Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes), the BD-exposed workers in the current study (both the monomer and polymer groups) did not show significant increases in the frequencies of *HPRT* mutations. Nevertheless, it was important to determine if differences in the spectra of mutations in the different groups could indicate genetic effects from the BD exposure.

METHODS

Expansion of Clones

HPRT mutant isolates from the *HPRT* cloning assays reported in the previous section (Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes) were grown and then frozen for later molecular studies. Mutant clones were randomly selected, meaning that we did not exclusively select rapidly expanding clones. Among the clones selected, quite a few were very small from the beginning and did not expand substantially despite our efforts to provide the best possible growth conditions.

Mutant clones were expanded in the same medium that was used to select mutants except that recombinant IL-2 was replaced by 10% LAK supernatant (Tates et al 1991b) because, in addition to IL-2, the supernatant contained other growth factors that proved to be better for clonal expansion than recombinant IL-2 alone. Mutants were expanded by transferring the contents of one positive well to two wells without irradiated feeder cells. When a clone reached a size of approximately 30,000 cells, it was distributed over two wells with irradiated TK6 36X4 lymphoblastoid feeder cells or, depending on growth characteristics, all 30,000 cells were transferred to one well (2 cm^2) of a 24-well plate.

Because clones often had widely different growth characteristics, the expansion procedure for a particular clone depended largely on its growth. Some clones could easily be expanded; for others, the expansion was cumbersome or impossible. Expanded clones were usually transferred to four 1.5-mL Eppendorf tubes with 500 μL RPMI 1640 medium and centrifuged at 17,000*g* for 15 seconds in a fixed-angle Eppendorf centrifuge. The supernatant was discarded, and the tubes containing the cell pellet were frozen and stored at −80°C. Approximately 10 to 20% of the isolates consisted of not more than 30,000 to 50,000 cells, which meant that each tube contained about 7500 to 12,500 cells; other isolates reached larger cell numbers resulting in 30,000 to 50,000 cells per tube.

Up to twelve randomly selected mutants were frozen from each subject. Table 29 lists the number of *HPRT* mutant isolates per subject from the control, monomer, and polymer groups on which analysis was attempted. For most subjects, we also isolated, expanded, and froze five wild type clones; these were used, among other things, for determining metabolic genotypes (*CYP2E1*, *EH*, *GSTM1*, *GSTT1*, *ADH2*, *ADH3*; also see the section Workers' Exposure Groups, Personal Characteristics, and Metabolic Genotypes / Metabolic Genotypes).

Table 28. Previous *HPRT* and *lac*I Mutation Results After BD, BDO, or BDO_2 Exposure[a]

	BD			BDO			BDO_2			
Mutation Type	In Vivo Mice Exon 3 Only[b]	In Vivo Mice RT Analysis[b]	In Vivo Humans[c]	In Vitro TK6 Cell Line[d]	In Vitro TK6 Cell Line[e]	In Vitro *lac*I/Rat2 Cells[e]	In Vitro TK6 Cell Line[c]	In Vitro TK6 Cell Line[e]	In Vivo Mice Exon 3 Only[b]	In Vivo Rats (Compared with BD)[b]
Transitions										
G•C→A•T	↓↓	NC	NC	ND	↑	↑ (non CpG)	ND	↓↓	↑	↑↑
A•T→G•C	NC	↑↑	↑	ND	↑↑	NC	ND	↓	↑	NC
Transversions										
A•T→T•A	NC	↓↓	↑↑	ND	↑↑	↑↑	ND	↑↑	NC	↑
A•T→C•G	↑↑	↑	↑	ND	NC	↑↑	ND	↓	↑	NC
Frameshifts	NC in −1 ↓ in +1	NC in −1 ↑ in +1	↑↑ in −1	ND	Not reported	↓ in −1 and +1	ND	Not reported	↓ in +1	↑ in −1 ↓↓ in +1
5′ Deletions	↓ in small deletions	↑↑ in exon exclusions (may be deletions)	↑ in small deletions	NC	↓	↓ in small deletions	↑	↑↑	↓ in small deletions	↓ in small deletions
3′ Deletions	↓ in small deletions	↑↑ in exon exclusions (may be deletions)	↑ in small deletions	NC	↓↓	↓ in small deletions	↑	↓	↓ in small deletions	↓ in small deletions

[a] Change in frequency noted as: ↑ = increase; ↓ = decrease; double arrows equal large increase or decrease; NC = no change; ND = not determined.

[b] Walker and Meng 2000.

[c] Ma et al 2000.

[d] Cochrane and Skopek 1994.

[e] Steen et al 1997a,b; Recio et al 2000.

Table 29. Summary of *HPRT* Mutant Isolates from Study Subjects

Subject Number	*HPRT* Isolates	Positive RT-PCR[a]	Multiplex-PCR[b]
Administrative Control Subjects (n = 23[c])			
108	9	2	4
109	4	1	1
114	5	1	2
115	12	2	3
116	11	5	2
118	11	5[d]	2
119	12	1	3
120	7	2	2
121	11	4	6
122	5	2	1
123	11	5[e]	5
155	1	1	0
156	3	0	2
166	4	1	1
167	2	0	1
168	12	2	7[f]
169	1	0	1
170	3	0	2
171	3	0	2[f]
172	2	1	1
182	5	2	2
183	4	1	2
184	12	2	2
Total	150	40	54 (15 with size change or loss)
Percentage of isolates[g]		26.7	36.0
Monomer Production Workers (n = 18[c])			
102	12	2	4
103	12	3	7
105	12	4	6
113	12	4	5
124	12	4	3
125	12	1	6
126	4	0	1
127	12	3	5
128	6	2	3
141	2	0	1
142	7	3	1
143	10	1	4
149	12	0	4
150	2	0	0
151	4	1	1
152	6	5	0
157	3	2	1
177	2	0	1
Total	142	35	53 (20 with size change or loss)
Percentage of isolates[h]		24.7	37.3

(*Table continues next column*)

Table 29 (*Continued*). Summary of *HPRT* Mutant Isolates from Study Subjects

Subject Number	*HPRT* Isolates	Positive RT-PCR[a]	Multiplex-PCR[b]
Polymerization Workers (n = 28[c])			
107	1	0	1
111	4	0	1
112	1	1	0
129	7	3	2
130	3	1	1
131	12	1	5
132	8	0	3[f]
133	3	0	1
134	5	2[d]	2
135	2	0	0
137	9	5[i]	3
138	4	1	3
139	12	1	7
145	8	4[j]	2[j]
146	10	3	3
154	12	1	5
158	7	1	3
159	2	2	0
160	1	0	0
162	5	2[i]	3
164	5	2	2
165	2	0	1
173	4	0	4
174	3	1	0
175	1	0	0
176	6	1	3
179	8	0	2
180	4	1	2
Total	149	33	59 (10 with size change or loss)
Percentage of isolates[k]		22.2	39.6

[a] RT-PCR used for molecular studies of *HPRT* mRNA.

[b] Multiple PCRs of genomic DNA used for molecular studies of *HPRT* DNA.

[c] For several reasons (described in the text), it proved impossible to expand mutant clones from two administrative control subjects, five monomer production workers, and six polymerization workers. In addition, the monomer group had one less subject because the blood sample was not shipped from Prague to Leiden for analysis.

[d] Two isolates had the same *HPRT* mutation by RT-PCR.

[e] Five isolates had the same *HPRT* mutation by RT-PCR.

[f] Two isolates had the same *HPRT* mutation by multiplex-PCR.

[g] Of all *HPRT* mutant isolates from administrative control subjects, 63% [(40 + 54)/150] were analyzed.

[h] Of all *HPRT* mutant isolates from monomer production workers, 62% [(35 + 53)/142] were analyzed.

[i] Three isolates had the same *HPRT* mutation by RT-PCR.

[j] Three isolates had the same mutation, two by RT-PCR and one by multiplex-PCR.

[k] Of all *HPRT* mutant isolates from polymerization workers, 62% [(33 + 59)/149] were analyzed.

PCR Amplification and DNA Sequencing

Our usual procedure for synthesizing complementary DNA (cDNA) from messenger RNA (mRNA) is to resuspend a dry cell pellet into a lysis buffer. However, the samples in the current study were not completely dry when quickly frozen, which made lysing the cells for cDNA synthesis problematic. To use these cell aliquots, the samples were quickly defrosted, the cells centrifuged, the liquid removed, and the lysis buffer added. During this procedure some mRNA (and DNA) may have been lost into the discarded supernatant; this resulted in failure of the subsequent RT-PCR or multiplex-PCR of genomic DNA. In addition, some samples contained few cells. For these reasons, some mutant samples could not be analyzed, but the complicating factors and sample losses were equally distributed among the exposure groups (see Table 29). In each of the three exposure groups, approximately 25% of the *HPRT* isolates produced a positive RT-PCR and approximately 38% produced a successful multiplex-PCR.

RT-PCR and cDNA sequencing of the *HPRT* isolates was based on the method of Yang and colleagues (1989) with modifications. To perform the RT step, 18 μL of 5.6 mM $MgCl_2$, 1.1× PerkinElmer Buffer II, 2.78% Nonidet P 40, 0.56 mM of each of four dNTPs, 2.78 μM oligo dT (PerkinElmer), 1.1 U RNase inhibitor (PerkinElmer), and 2.78 U MuLV (PerkinElmer) were added to the centrifuged cell pellet. This was then maintained at 25°C for 10 minutes, at 37°C for 25 minutes, at 99°C for 5 minutes, and then at 4°C. A nested PCR of the room-temperature product then followed. The first round of PCR consisted of 5 μL of room-temperature product plus 19.875 μL of 1.26 mM $MgCl_2$, 1.0× PerkinElmer Buffer II, 0.25 mM of each of four dNTPs, 0.16 μM primer SJ-A (CGCCC AAAGG GAACT GATAG TCTAT AGGC), and 0.16 μM primer SJ-B (CCTCT GCTCC GCCAC CG). After 5 minutes at 94°C, 0.125 μL of *Taq* polymerase (Sigma) was added and the PCR profile was 94°C for 1 minute, 65°C for 1 minute, and 72°C for 2 minutes for 30 cycles, followed by 72°C for 7 minutes and 4°C. For the second round of PCR, 1 μL of first-round product was added to 49 μL of 1.53 mM $MgCl_2$, 1.0× PerkinElmer Buffer II, 0.20 mM of each of four dNTPs, 0.13 μM primer B (CCTGA GCAGT CAGCC CGCGC), and 0.13 μM primer 4b (GCAAA AAGCT CTACT AAGCA GATGG CCACA G). The reaction profile was 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute for 30 cycles, followed by 72°C for 7 minutes and 4°C. The product was run on a 1% agarose gel and the largest band was excised and cleaned with GENECLEAN (Qbiogene).

The RT-PCR products were sequenced with primer B (antisense), which usually produced the sequence of the entire *HPRT* 657-bp coding region. If the sequence of the entire coding region was not readable or if a mutation was not found, then the primer A (sense: CAATA GGACT CCAGA TGTTT) was used for sequencing in the opposite direction.

Direct sequencing of PCR products was performed at the Vermont Cancer Center DNA Analysis Facility (Burlington VT, USA). Briefly, a BigDye terminator cycle sequencing ready-reaction kit (Applied Biosystems) was used to amplify the signal for automated sequencing. With this kit, the dyes are attached to the dyedeoxy terminators. The sequencing premix was diluted 2:1 with 5× sequencing buffer and 6 μL was used along with ~75 ng template DNA, 3.2 pmol primer, and brought to a 20-μL volume with water. The reaction mixture underwent one round of 25 cycles at 96°C for 30 seconds, a rapid thermal ramp to 50°C and held for 15 seconds, and then a rapid thermal ramp to 60°C and held for 4 minutes. Cycle sequencing extension products were purified, dried, resuspended in a 5:1 mixture of formamide and ethylenediaminetetraacetic acid (EDTA), loaded on a stretch sequencer (Applied Biosystems 373XL), and analyzed with Sequence Analysis V3.0.

Those mutants that did not result in an RT-PCR product were then analyzed by multiplex-PCR of genomic DNA. The simultaneous amplification of all nine *HPRT* exons can be achieved using eight pairs of oligonucleotides in a single PCR (Gibbs et al 1990); however, because the DNA may not have been in perfect condition for the reasons given above, the multiplex mixture was divided into two sets, one consisting of exons 1, 2, 4, and 6 (set 1) and the other containing exons 3, 5, 7/8, and 9 (set 2). The pellet remaining after RT-PCR was lysed using 10 μL 0.05% Tween 20, 0.05% NP40, and 0.1 mg/mL proteinase K, and incubated for 1 hour at 56°C followed by 10 minutes at 96°C. A 2-μL portion of the lysis mixture was amplified with primers for either set 1 or set 2 in a total volume of 25 μL containing 6.7 mM $MgCL_2$, 16.6 mM ammonium sulfate $[(NH_4)_2SO_4]$, 5 mM 2-mercaptoethanol, 6.8 μM EDTA, 67 mM Tris HCl (pH 8.8 at 25°C), 1.5 mM of each of four dNTPs, and 10% DMSO. The final primer amounts were 10 pmol of each primer for exons 4 and 5; 20 pmol of each primer for exons 1, 2, 3, 6, and 9; and 25 pmol of each primer for exons 7/8. The reactions were overlaid with mineral oil and heated to 94°C for 5 minutes; 0.4 μL *Taq* polymerase (Gibco) was added (hot start) followed by 35 cycles of DNA polymerization (68°C for 2 minutes), denaturation (94°C for 30 seconds), and annealing (61°C for 50 seconds). The final incubation was 68°C for 7 minutes. Products were run on a 1.4% agarose gel containing ethidium bromide and photographed on a transilluminator. The appearance of the exon 7/8 product was variable; therefore, exon 7/8 was not scored in the multiplex analysis. Exon products that had shifted from their expected position

were cut out and purified by Qiaquick columns (Qiagen) or by GENECLEAN for DNA sequencing. No further analyses were done on exons that had not shifted.

For mutants that showed loss of one or more exons in their RT-PCR product, the RT-PCR leftover pellet was also lysed as above and the genomic DNA was amplified by PCR for the relevant exon or exons, as we had done for multiplex-PCR. This was done to determine if the mutation was a deletion or a splice mutation. If the relevant exon or exons were present in genomic DNA, the exon products were purified as above and sequenced to determine the mutation.

For some mutants with indeterminate results (eg, possible total deletions), an additional multiplex-PCR was performed under the same conditions described above using exons 1, 4, and 9, and dystrophin as a positive control.

Statistical Analysis

The distributions of different types of mutations in the three exposure groups were compared by χ^2 analysis.

RESULTS

Although more than 140 *HPRT* mutant isolates were obtained from individuals in each of the three exposure groups (441 total mutant isolates), information on many of those was not obtainable for the technical reasons given in the earlier section Biomarkers of Effect: *HPRT* Mutational Spectra / Methods / Expansion of Clones. Analyses by RT-PCR and sequencing were attempted for all isolates, but a cDNA product was amplified and sequenced for only 108 (24% of the total).

RT-PCR provides a cDNA to analyze the genetic alteration at the mRNA level, which is usually sufficient to infer the alteration at the DNA level. The exceptions are (1) when no cDNA is produced because of a genomic rearrangement or promoter mutation or (2) when there are exclusions of one or more exons in the cDNA where a number of alterations in genomic DNA could be the basis of the change. PCR of the relevant genomic regions in these latter mutants was then done for sequence analysis to define the cause of the exclusion. Multiplex-PCR analysis was used to amplify the genomic DNA of all remaining mutant isolates (333) that had not yielded an RT-PCR product; this was successful for 166 of the 333 remaining isolates. However, sequence analyses were done only for those exonic regions that showed an alteration in size from wild type. Only 45 of the 166 (27%) mutant isolates showed multiplex-PCR products with size changes or losses. The remaining 121 that showed no changes at this level of study were almost certainly base-substitution mutations that were not detected in cDNA because no product had been formed. Analyzing all nine exons for this many isolates (121) was not justifiable. Therefore, these isolates were included in calculations of the frequencies of deletions but not of the frequencies of point mutations (these mutants are listed as "no change" in Table 31).

Table 30 lists the results of the RT-PCR analyses. In this table, mutant isolates from the same individual that showed the same molecular change (eg, the same base substitution at the same position) were considered to be "sibling" mutants and were entered only once. Sibling mutants are simply replicates of the same in vivo mutational event that arose by in vivo clonal amplification. By χ^2 analysis, we found no significant differences in the distributions of the kinds of mutations among the control, monomer, and polymer groups. Specifically, the monomer and polymer groups did not show the signature A•T→T•A transversions in a larger percentage than that found in the control group; in fact, the most heavily exposed polymer group showed none. Similarly, we found no significant increases in the percentages of A•T→G•C or G•C→A•T transitions in either of the exposed groups compared with the control group. The percentages of total transitions in the monomer and polymer groups were greater than the percentages of total transversions in the same groups; whereas in the control group, the reverse was seen (total transversions were greater than total transitions). However, transitions in the control group were lower than we expected on the basis of our laboratory's historical control groups and a summary of the background *HPRT* mutational spectra in normal individuals (Curry et al 1999), where transitions and transversions were found to be equal.

The monomer group had a greater percentage of frameshift mutations than did either of the other groups; that is, 11% versus 2.9% in the control group and none in the polymer group. All of the frameshift mutations were −1, which is the loss of a single base pair. The monomer and polymer groups had higher percentages of deletions (small and large deletions plus small deletions/insertions) than the control group, that is, 25.5% and 24.7% vs 22.4%, respectively. None of these differences among groups, however, was statistically significant.

The results of the multiplex-PCR analyses of genomic DNA for the mutant isolates that did not yield an RT-PCR product are given in Table 31. Of the mutant isolates analyzed, 121 yielded multiplex-PCR products with no size change compared with wild type and were not analyzed further. As stated before, all of these would probably have shown single base substitutions if they had been sequenced. Assuming this to be true, the monomer group had a higher

Table 30. RT-PCR Mutation Analysis Results[a]

	Control		Monomer		Polymer	
	n (%)[b]	*n* (%)[c]	*n* (%)[b]	*n* (%)[c]	*n* (%)[b]	*n* (%)[c]
Isolates analyzed	40		35		33	
Total independent mutations	35 (100)		35 (100)		28 (100)	
Mutation type						
Transitions[d]	8 (22.9)		11 (31.4)		8 (28.6)	
G•C→A•T		4 (50.0)[d]		7 (63.6)		5 (62.5)
A•T→G•C		4 (50.0)		4 (36.4)		3 (37.5)
Transversions[d]	14(40.0)		9 (25.7)		7 (25.0)	
G•C→C•G		6 (42.9)		3 (33.0)[e]		1 (14.3)
G•C→T•A		1 (7.1)		2 (22.2)		2 (28.6)[d]
A•T→T•A		4 (28.6)		3 (33.0)		0 (0)
A•T→C•G		3 (21.4)[d]		1 (11.1)		4 (57.1)[d]
Small deletions[f]	4 (11.4)		3 (8.6)		4 (14.3)[g]	
Small deletions and insertions	2 (5.7)		1 (2.9)		1 (3.6)	
Small insertions	0 (0)		0 (0)		1 (3.6)	
Large deletions[h]	2 (5.7)		5 (14.3)		2 (7.1)	
Frameshifts	1 (2.9)		4 (11.4)		0 (0)	
Single-exon or multiple-exon exclusions in cDNA but no genomic deletion	3 (8.6)		1 (2.9)		1 (3.6)	
No mutation found	1 (2.9)		1 (2.9)		4 (14.3)	

[a] Exon deletions were confirmed by multiplex-PCR analysis of genomic DNA when necessary to differentiate from exon exclusions due to splicing errors.

[b] Values in parentheses are percentages of the *Total independent mutations* (given in the second row of the table).

[c] For categories of transitions and transversions, the percentages of *Transitions* or *Transversions* are shown in parentheses. The category values are offset to the right of the main column so they are not added twice into the *Total independent mutations*.

[d] Sibling mutations; only one is listed for each set.

[e] One mutation was at a CpG site.

[f] Small deletions involve loss of 50 bp or loss of genomic DNA and do not include an entire exon.

[g] Two pairs of sibling mutations; only one is listed for each pair.

[h] Large deletions involve loss of one or more exons due to loss of genomic DNA.

Table 31. Multiplex-PCR Mutation Analysis Results[a]

	Control *n* (%)	Monomer *n* (%)	Polymer *n* (%)
Clones analyzed	54	53	59
Total independent mutations	52 (100)	53 (100)	58 (100)
Mutation type			
Total-gene deletions (exons 1—9)	3 (5.8)[b]	5 (9.4)	2 (3.4)[b]
Large deletions (≥ 1 exon but < 9 exons)	7 (13.5)[b]	11 (20.8)	5 (8.6)
Small deletions (< 50 bp)	3 (5.8)	4 (7.5)	2 (3.4)
No change	39 (75.0)	33 (62.3)	49 (84.5)

[a] Values in parentheses are percentages of the *Total independent mutations* (given in the second row of the table).

[b] Pair of sibling mutations; only one is listed for the pair.

percentage of any kind of deletion mutation (20/53 = 38%) than did the control group (13/52 = 25%) or the polymer group (9/58 = 16%) by this analysis. The most heavily exposed polymer group had the lowest percentage of deletion mutations. None of the differences among groups shown in Table 31 was statistically significant.

The specific base-substitution mutations seen in this study are given in Table 32. Several have not been previously reported; these include: in the control group, the 27G→C, 49T→G, 76A→G, and IVS4–1G→A mutations; in the monomer group, the 624T→C mutation; and in the polymer group, the 233T→G and 409A→G mutations. Some base-substitution mutations were seen several times in different individuals:

- 197G→A was seen three times in the monomer group and once in the polymer group; it is a known *HPRT* "warm spot," that is, a site where mutations occur somewhat more frequently than the average.
- The 496A→G mutation was seen once in the monomer group and once in the polymer group.
- Mutations at position 602 were seen three times (A→G in a monomer production worker, and A→T in a monomer production worker and in an administrative control subject); although mutations have been previously observed at this site, it is not a known mutational warm spot.
- The 551C→G mutation (in both the control and monomer groups) is a known mutational warm spot.
- One mutation was seen at position 568 (G→A in one polymerization worker).
- Five mutations were also seen at position 611 (an A→T in one administrative control subject; and an A→G in one administrative control subject, two monomer production workers, and one polymerization worker); position 611 is a warm spot for A→G mutations.

Table 32. Point Mutations from RT-PCR Analysis

Mutant Name	Mutation[a]	Exon Affected	DNA Sequence Context[b]	Result in mRNA
Administrative Control Subjects				
118-3	1A→T	1	gtt ATG GCG	No ATG START
114-2	27G→C	1	GTC GTG gtg	Inclusion of IVS1+1 to IVS1+49
155-1	49T→G	2	GGT TAT GAC	Tyr17→Asp
168-12	76A→G	2	CCT AAT CAT	Asn26→Asp
184-11	135G→C	3	tgt agG ACT	Arg45→Ser
116-8	208G→C	3	AAG GGG GGC	Gly70→Arg
122-2	236T→C	3	CTG CTG GAT	Leu79→Pro
119-12	419G→A	6	ACT GGC AAA	Gly140→Asp
118-8, -9	494T→G	7	CTG GTG AAA	Val165→Gly
116-3	494T→G	7	CTG GTG AAA	Val165→Gly
120-4	529G→T	7	CCA GAC Tgt	Asp177→Tyr
116-4	533T→C	8	tta gTT GTT	Phe178→Ser
168-7	551C→G	8	ATT CCA GAC	Pro184→Arg
115-12	595T→A	8	TAC TTC AGG	Phe199→Ile
183-2	602A→T	8	AGG GAT TTG	Asp201→Val
115-11	610C→G	9	tag CAT GTT	His204→Asp
172-2	611A→T	9	tag CAT GTT	His204→Leu
121-7	611A→G	9	tag CAT GTT	His204→Arg
121-4	IVS3–1 g→c	3	aac tag AAT	Loss of exon 4
116-9	IVS4+1 g→a	4	AAG gta tgt	Loss of exon 4
123-1, -2, -4, -7, -10	IVS4–1 g→a	5	ttc tag AAT	Loss of exon 5
121-8	IVS8+5 g→a	8	gta agt aat	Loss of exon 8

(Table continues next page)

[a] IVS (intervening sequence) = intron; eg, IVS4+1 is intron 4, 1st base from the 5′ end (beginning), and IVS3–1 is intron 3, 1st base from the 3′ end (end).

[b] Uppercase letters are exon sequences; lowercase letters are intron sequences.

Table 32 (*Continued*). Point Mutations from RT-PCR Analysis

Mutant Name	Mutation[a]	Exon Affected	DNA Sequence Context[b]	Result in mRNA
Monomer Production Workers				
143-5	59A→T	2	CTT GAT TTA	Asp20→Val
124-11	152G→C	3	GCT CGA GAT	Arg51→Pro
124-10	197G→A	3	CTC TGT GTG	Cys66→Tyr
128-5	197G→A	3	CTC TGT GTG	Cys66→Tyr
142-7	197G→A	3	CTC TGT GTG	Cys66→Tyr
124-1	200T→G	3	TGT GTG CTC	Val67→Gly
152-4	388G→T	5	AAT GTC TTG	Val130→Phe
157-2	459T→A	6	CAG TAT AAT	Tyr153→STOP
127-9	496A→G	7	GTG AAA AGG	Lys166→Glu
152-3	538G→A	8	GTT GGA TTT	Gly180→Arg
128-6	551C→G	8	ATT CCA GAC	Pro184→Arg
113-2	569G→T	8	GTA GGA TAT	Gly190→Val
113-12	569G→C	8	GTA GGA TAT	Gly190→Ala
142-2	569G→A	8	GTA GGA TAT	Gly190→Glu
113-7	602A→G	8	AGG GAT TTG	Asp201→Gly
103-4	602A→T	8	AGG GAT TTG	Asp201→Val
152-2	610C→T	9	tag CAT GTT	His204→Tyr
102-1	611A→G	9	tag CAT GTT	His204→Arg
142-1	611A→G	9	tag CAT GTT	His204→Arg
124-4	IVS1+5 g→a	1	gtg agc agc	Inclusion of IVS1+1 to IVS1+49
Polymerization Workers				
154-6	113C→T	2	ATT CCT CAT	Pro38→Leu
129-2	134G→A	2	GAC AGg taa	Arg45→Lys
174-1	148G→C	3	CTT GCT CGA	Ala50→Pro
180-3	197G→A	3	CTC TGT GTG	Cys66→Tyr
146-10	233T→G	3	GAC CTG CTG	Leu78→Arg
134-4, -5	389T→G	5	AAT GTC TTG	Val130→Gly
137-6	409A→G	6	ATA ATT GAC	Ile137→Val
159-2	496A→G	7	GTG AAA AGG	Lys166→Glu
139-5	548T→G	7	GAA ATT CCA	Ile183→Ser
129-4	568G→A	7	GTA GGA TAT	Gly190→Arg
131-7	611A→G	9	tag CAT GTT	His204→Arg
146-2	617G→A	9	GTT TGT GTC	Cys206→Tyr
138-1	IVS3+1 g→t	3	tgt agG ACT	Loss of exons 2 and 3
145-1, -3, -8	IVS7–1 g→t	8	tta gTT GTT	Inclusion of IVS2–2 and IVS2–1; add 7 amino acids
137-1	IVS8–3 t→g	9	tta tag CAT	Inclusion of IVS8–1 to IVS8–2; STOP at new amino acid 251

[a] IVS (intervening sequence) = intron; eg, IVS4+1 is intron 4, 1st base from the 5′ end (beginning), and IVS3–1 is intron 3, 1st base from the 3′ end (end).

[b] Uppercase letters are exon sequences; lowercase letters are intron sequences.

- Three mutations were seen at position 569 in the monomer group (G→T, G→A, G→C); mutations have previously been observed here, but it is not considered a warm spot.
- Two mutations were seen at position 610 (C→G in an administrative control subject and C→T in a monomer production worker); a number of mutations have previously been observed at this site.
- Mutations at sites known as warm spots but seen only once or not at all in this analysis include: 3G→A, 143T→G or A, 146T→C or G, 151C→T, 508C→T (a CpG site), 568G→T or C or A, and 617G→A (seen in one polymerization worker).

In terms of sequence context, the G→A mutations in the control group occurred in the sequence context of A(or T)G(or A)GNA (where N designates any base), whereas the G→A mutations in the monomer and polymer groups arose mostly in the C(or T)TGG(or T)N sequence context. A→T mutations arose mostly in a consensus sequence of G(or T)G(or T)ATT.

Table 33 shows the five frameshift mutations found in this study. All were one-base deletions. Two of the five frameshifts were not in sequence runs of a single nucleotide and none were in the common "hot spot" (a site of very frequent mutations), that is, the sequence of six Gs in exon 3. Frameshift mutations have been reported at two of the sites found in this study (ie, those in exons 4 and 8); however, the previously reported occurrences were +1 frameshift rather than the −1 frameshift mutations seen here.

Table 34 lists all the small deletions, deletion/insertions, and insertion mutations observed by either RT-PCR or multiplex-PCR analyses. Small deletions and insertions are arbitrarily defined by the investigator; here, the terms are used to mean gains or losses of bases in genomic DNA that are not large enough to include entire exons, even though exons may be excluded from cDNA due to changes in splicing caused by the genomic loss or gain. None of the mutations we found has been reported previously. In Table 34, those mutations with the mutant name shown in boldface, italic, large type were discovered by multiplex-PCR analyses of genomic DNA; those in normal type were discovered during RT-PCR analyses of cDNA. One point of interest is that many of these small deletions/insertions were ascertained as small gel shifts of multiplex-PCR products, which in our experience is an unusual occurrence. Four of the nine mutations in administrative control subjects, three of the eight mutations in monomer production workers, and four of the six mutations in polymerization workers have one or more repeated bases at the breakpoints (underlined in the DNA Sequence Context column of Table 34).

Table 35 lists the large deletions seen in this study. Large deletions are those mutations that show genomic DNA losses of sufficient size to delete one or more exons. Although more large deletions were seen in the monomer group than in the other two exposure groups, they were not of any specific type and the differences in percentages between exposure groups were not significant.

Table 36 lists the undefined mutations. Some show that one or more exons were excluded in cDNA but no apparent deletions in the genomic PCR product. Others showed wild type sequence in the RT-PCR product but no further analyses were done. The reasons for the exon exclusion mutations shown here are unknown but may be due to an inversion or a point mutation (possibly a STOP mutation) in one of the excluded exons.

Table 33. Frameshift Mutations from RT-PCR Analysis

Mutant Name	Mutation[a]	Exon Affected	DNA Sequence Context[b]	Result in mRNA
Administrative Control Subjects				
122-4	555 del C	8	CCA GA<u>C</u> AAG	TAG at codon 189
Monomer Production Workers				
152-6	49 del T	2	CCA GG<u>T</u> TAT	TAA at codon 41
127-3	100 del A	2	TTG G<u>A</u>A AGG	TAA at codon 41
105-2	597 del C	8	TAC TT<u>C</u> AGG	TGA at codon 202
113-6	IVS4+1 del g	4	AAG <u>g</u>ta tgt	Exclusion of exon 4

[a] IVS (intervening sequence) = intron; eg, IVS4+1 is intron 4, 1st base from the 5′ end (beginning), and IVS3–1 is intron 3, 1st base from the 3′ end (end).

[b] Uppercase letters are exon sequences; lowercase letters are intron sequences.

Table 34. Small Deletions, Deletions and Insertions, and Insertions from RT-PCR and Multiplex-PCR Analyses

Mutant Name[a]	Mutation	Exon Affected	DNA Sequence Context (Deleted) [Inserted][b]	Result in cDNA[c]
Administrative Control Subjects				
183-3	Del 37 to 78	2	AGT GAT (GAT GAA ... ... CCT AAT) CAT TAT	Del 42 bp; loss of 14 amino acids
108-4	Del 85 to 114	2	CAT TAT (GCT GAG ... ... ATT CCT) CAT GGA	Del 30 bp; loss of 10 amino acids
108-1	Del IVS1–20 to IVS1–4; ins aagctg	2	ttgaaa(cag cta ... ... ctt ttt) [aagctg] cag ATT	Del 17 bp; ins 6 bp; exclusion of exon 2
108-7	Del IVS1–11 to IVS1–30	2	gctatat (ttt ctt ... ... cag ATT) AGT GAT	Exclusion of exon 2
118-1	Del 163 to 165	3	GTG ATG (AAG) GAG ATG	Del 3 bp; del Lys55 codon
168-4	Del IVS4–16 to IVS5+42; ins a	5	ttc att (tct ctt ... ... ctt ttc) [a] taa ctt	Del 76 bp; ins 1 bp
118-4	Del IVS7+5 to IVS7+11; ins at	7	GAC Tgt aa(g tgaatt) [at] act ttt	Del 7 bp; ins 2 bp; exclusion of exon 7
182-1	Del 552 to 589	8	ATT CC(A GAC AAG ... ... AAT G)AA TAC TTC	Del 38 bp; TAG at new codon 197
109-3	Del IVS7–20 to IVS7–13	8	aaatga (tga att at)g att ctt	Del 8 bp; exclusion of exon 8
Monomer Production Workers				
105-3	Del 4 to 14	1	gtt ATG (GCG ACC CGC AG)C CCT	Del 11 bp; loss of 3 amino acids; TAG at new codon 7
152-5	Del 69 to 96	2	TTT TG(C ATA CCT ... ... GAT TTG) GAA AGG	Del 28 bp; loss of 9 amino acids; TAA at new codon 32
105-12	Del 183 to 185	3	GGC CAT CA(C AT)t gta gcc	Del 3 bp; del Ile62 codon
113-9	Del 218 to 299	3	GGC TAT A(AA TTC ... ... TTT AT)C AGA CTG	Del 82 bp; loss of 27 amino acids; TGA at new codon 75
105-1	Del IVS2–39 to IVS2–154	3	taa tgt (atg aaa ... ... CGA G)AT GTG	Del 59 bp; unknown
128-3	Del 402 to IVS5+29	5	GTG GA(A gta agt ... ... ata aca)ttt atga	Del 30 bp; unknown
127-6	Del 436 to 469; ins G	6	CAG ACT (TTG CTT ... ... CCA AAG A)[G] TG GTC	Del 34 bp; ins 1 bp; loss of 11 amino acids
102-2	Del 458 to IVS6+14; ins a	6	AGG CAG TA(T ... ... gac att) [a] ttg aca	Del 44 bp; unknown
Polymerization Workers[d]				
137-2, ***-3***, -9	Del 51 to 76	2	CCA GGT TA(T GAC ... ... ATA CCT A)AT CAT	Del 26 bp; immediate TAA at codon 17
162-1, -2, -4	Del IVS1–1 to IVS1–50	2	ttt ca(g ATT AGT ... ... GGT TA)T GAC CTT	Del 24 bp; exclusion of exon 2
130-1	Del 431 to 478; ins CGACTT	6	ACA ATG C(AG ACT ... ... AAG G)[CGACTT]TC GCA	Del 48 bp; Gln144→Pro codon; Leu146→ Phe codon; loss of 14 amino acids
164-4	Del IVS6–15 to IVS6–2	7	tca ca(t ttt gta att aac a)gC TTG CTG	Del 14 bp; exclusion of exon 7
158-5	Del IVS8+5 to IVS8+8	8	AAT gta a(gt aa)t tgc ttc	Del 4 bp; exclusion of exon 8
145-7	IVS7–1 to IVS7–2; ins ttt	8	ttta[ttt] gTT GTT GGA	Exclusion of exon 8

[a] Mutant names in bold, italic, large type, are from multiplex-PCR analyses; those in normal type are from RT-PCR analyses.

[b] Repeated bases at the breakpoints are underlined. Uppercase letters are exon sequences; lowercase letters are intron sequences.

[c] Exclusion = loss of indicated exon from cDNA product; TAG and TAA are STOP codons.

[d] Six deletions or deletions and insertions were observed for the polymerization workers. Two of these included replicate sibling mutants (triplicates), two of which were characterized by RT-PCR analysis (Table 30) and the other single mutant by multiplex-PCR analysis (Table 31).

Table 35. Large Deletions from RT-PCR and Multiplex-PCR Analyses

Mutant Name[a]	Mutation
Administrative Control Subjects	
171-1, -3	Del exons 1 to 9
109-2	Del exons 1 to 9
168-10	Del exons 1 to 9
115-9	Del exon 1
156-1	Del exon 4
121-5	Del exon 5
168-5, -8	Del exon 5
166-4	Del exon 4[b]
170-1	Del exons 2 to 9
167-2	Del exons 5 to 9
108-9	Del exons 6 to 9
108-6	Del exon 9
Monomer Production Workers	
177-3	Del exons 1 to 9
105-9	Del exons 1 to 9
103-1	Del exons 1 to 9
149-4	Del exons 1 to 9
102-6	Del exons 1 to 9
124-7	Del exon 1
149-8	Del exons 1 to 3, 5 to 9
103-11	Del exon 3
125-11	Del exon 4
105-10	Del exon 4
127-11	Del exon 5
113-8	Del exon 6
151-4	Del exons 2 to 3
105-8	Del exons 2 to 3
102-11	Del exons 2 to 3
125-6	Del exons 2 to 5
125-1	Del exons 4 to 5
125-10	Del exons 4 to 9
105-5	Del exons 4 to 9
127-8	Del exons 5 to 9
149-2	Del exons 7 to 9
Polymerization Workers	
132-3, -4	Del exons 1 to 9
173-3	Del exons 1 to 9
146-2	Del exon 1
129-3	Del exon 2
164-5	Del exon 4
111-2	Del exon 4
154-4	Del exon 4
131-9	Del exons 2 to 6
176-3	Del exons 4 to 9

[a] Mutant names in bold, italic, large type are from the multiplex-PCR analyses; those in normal type are from RT-PCR analyses.

[b] This mutation had a very unusual cDNA. Exon 1 was followed by bases 28 to 46 of exon 2, then intron 6 bases 31685 to 31701, then exons 6 to 9. Apparently, splicing occurred at a cryptic donor site in exon 2 to a cryptic "new" exon 5, which then spliced to exon 6. Multiplex-PCR analysis showed that exons 2, 3, and 5 were present; it is unknown why they were not spliced in correctly.

Table 37 summarizes the results of RT-PCR and multiplex-PCR analyses combined, both without and with partial correction for clonality. Clonality produces multiple mutant isolates showing the same mutational change that represents only a single mutational event. For example, individual 118 (an administrative control subject) showed two mutant isolates with the same mutation (494T→G) and individual 123 (also an administrative control subject) showed five mutant isolates with the same mutation, IVS4–1G→A (Table 32). Three individuals from the polymer group (subjects 145, 137, and 162) each showed three mutant isolates with the same mutational change; that is, IVS7–1G→T, del 51 to 76, and del IVS1–1 to IVS1–50, respectively (Table 32 for point mutations and Table 34 for deletions). Several additional mutants were pairs. Table 37 shows that the monomer group had a greater proportion of large deletions than the other groups, but this difference was not statistically significant and the rest of the distribution was similar among the groups.

DISCUSSION

We did not find an increase in A•T→T•A transversions among the *HPRT* mutations in BD-exposed workers. This was the specific mutational change observed in a number of other studies, both in vitro and in vivo (Ma et al 2000; Recio et al 2000). Of particular note, Ma and associates (2000) reported a study of BD-exposed individuals in which they found an increase in A•T→T•A transversions

Table 36. Undefined Mutations

Mutant Name	Mutation[a]
Administrative Control Subjects	
116-5	Wild type; identified by RT-PCR
184-12	Exclusion of exons 2 to 3; wild type, genomic
120-7	Exclusion of exons 2 to 8; wild type, genomic
182-3	Exclusion of exons 4 to 8; wild type, genomic
Monomer Production Workers	
103-10	Wild type; identified by RT-PCR
157-3	Exclusion of exon 8; wild type, genomic
Polymerization Workers	
137-8	Wild type; identified by RT-PCR
112-1	Wild type; identified by RT-PCR
159-1	Wild type; identified by RT-PCR
146-9	Wild type; identified by RT-PCR
176-6	Exclusion of exons 2 to 6; wild type, genomic

[a] Wild type indicates no change from normal sequence; exclusion indicates exons excluded from cDNA product.

Table 37. Summary of RT-PCR and Multiplex-PCR Results

	Control		Monomer		Polymer	
Mutation Type	*n* (%)[a]	*n* (%)[b]	*n* (%)[a]	*n* (%)[b]	*n* (%)[a]	*n* (%)[b]
No Correction for Clonal Expansion						
Total isolates and clones analyzed	94 (100)		88 (100)		92 (100)	
Point mutations	67 (71.3)		57 (64.8)		67 (72.8)	
RT-PCR		28 (41.8)		24 (42.1)		18 (26.9)
No changes on multiplex[c]		39 (52.2)		33 (57.9)		49 (73.1)
Small deletions	6 (6.4)		6 (6.8)		8 (8.7)	
Small deletions and insertions	3 (3.2)		2 (2.3)		1 (1.1)	
Small insertions	0 (0)		0 (0)		1 (1.1)	
Total-gene deletions	4 (4.3)		6 (6.8)		3 (3.3)	
Large 5′ deletions	1 (1.1)		1 (1.1)		1 (1.1)	
Large 3′ deletions	4 (4.3)		4 (4.5)		1 (1.1)	
Large internal deletions	5 (5.3)		10 (11.4)		5 (5.4)	
Undefined or no change on RT-PCR	4 (4.3)		2 (2.3)		5 (5.4)	
Partial Correction for Clonal Expansion						
Total isolates and clones analyzed	87 (100)		88 (100)		84 (100)	
Point mutations	62 (71.3)		57 (64.8)		64 (76.2)	
RT-PCR		23 (37.0)		24 (42.1)		15 (23.4)
No changes on multiplex[c]		39 (62.9)		33 (57.9)		48 (75.0)
Small deletions	6 (6.9)		6 (6.8)		4 (4.8)	
Small deletions and insertions	3 (3.4)		2 (2.3)		1 (1.2)	
Small insertions	0 (0)		0 (0)		1 (1.2)	
Total-gene deletions	3 (3.4)		6 (6.8)		2 (2.4)	
Large 5′ deletions	1 (1.1)		1 (1.1)		1 (1.2)	
Large 3′ deletions	4 (4.6)		4 (4.5)		1 (1.2)	
Large internal deletions	4 (4.6)		10 (11.4)		5 (6.0)	
Undefined or no change on RT-PCR	4 (4.6)		2 (2.3)		5 (6.0)	

[a] Values in parentheses are percentages of the *Total isolates and clones analyzed*.

[b] Values in parentheses are percentages of the *Point mutations*. The category values are offset to the right of the main column so they are not added twice into the *Total isolates and clones analyzed*.

[c] Presumed point mutations.

compared with the control group (University employees) (16% exposed group vs 8% control group). However, Walker and Meng (2000) found a decrease in *HPRT* A•T→T•A transversion mutations in mice exposed to BD in vivo. Our study did find a small but nonsignificant increase in G•C→A•T transition mutations in both the monomer and polymer groups. This increase has been reported in other BD studies (Recio et al 2000; Walker and Meng 2000), but the study by Ma and associates (2000) found no change in G•C→A•T transitions. A•T→G•C transition increases have also been observed in other studies (Ma et al 2000; Recio et al 2000).

BDO_2 is expected to induce deletion mutations because of its ability to form intrastrand and interstrand cross links. Although the monomer group in the current study did have an increased frequency of deletions as has been shown in other studies (Ma et al 2000; Recio et al 2000; Walker and Meng 2000), we did not observe an increase in the more highly exposed polymer group. The Ma and associates (2000) study found an increase of mutant isolates with total gene deletions (17.5% exposed group vs 9.7% control group) and isolates with multiple exon deletions (13.3% exposed group vs 6.5% control group). In addition, several of the deletion mutations observed in our study showed deletion breakpoints at sequences with one or more repeated bases. Such deletions are hypothesized to occur spontaneously due to slippage or homologous joining. For example, mutant 158-5 in a polymerization worker shows deletion of one gtaa in a gtaagtaa repeat (lowercase letters indicate intron sequences; uppercase letters indicate exon sequences); and mutant 152-5 in a monomer production worker shows a TTTG repeat both at the 5′ break-

point and at the end of the deletion. The mechanisms for the generation of the other deletions seen in this study are unclear; however, mutant 105-12 in a monomer production worker shows deletion of a CAT sequence within another CAT sequence, which could have arisen by slipping. One insertion mutation (145-7) was found in a polymerization worker, which showed an insertion of a TTT sequence in the splice acceptor site of exon 8. These deletion breakpoints all suggest deletions of spontaneous origin rather than due to BDO_2 interstrand or intrastrand linkage.

We found an increase in −1 frameshift mutations in the monomer group compared with the control group, but no frameshift mutations were seen in the polymer group. Other studies have shown mixed results with frameshift mutations; however the Ma and associates (2000) study showed an increase in −1 frameshifts (11% exposed vs 2% control group).

It is of interest to compare the mutational spectra determined here with those found in other relevant studies. Curry and associates (1999) have summarized the *HPRT* background mutational spectra in normal humans using data from 795 mutations from 24 published papers. Table 38 compares our BD control group (all males) to the Curry and colleagues spectrum for control males. These distributions do not differ significantly. All data from Ma and associates, Curry and colleagues, and the current study are given in Table 39 for comparison.

In summary, the molecular spectra of the *HPRT* mutant isolates recovered from both BD-exposed worker groups in this study were not statistically different from the molecular spectra of the mutants isolated from the unexposed control group. Further, all these spectra were statistically similar to what we and other researchers have observed historically for adults who have not been exposed to mutagens. Many of the deletion mutations observed in this study show breakpoints in sequences where spontaneous mechanisms for their origin can be postulated. The molecular mutational spectra identified in this study do not indicate BD-induced genetic effects in the workers.

Table 38. Comparison of Control Group Data from This Study with Historical Results from Molecular Analysis of Other Control Data (Curry et al 1999)[a]

	Control Data from This Study		Control Data from Curry et al	
	n(%)[b]	*n*(%)[c]	*n*(%)[b]	*n*(%)[c]
Total mutants analyzed	31		513	
Mutation type				
Transitions	8 (25.8)		188 (36.6)	
G•C→A•T		4 (50.0)		141 (75.0)
A•T→G•C		4 (50.0)		47 (25.0)
Transversions	14 (45.2)		172 (33.5)	
G•C→C•G		6 (42.9)		38 (22.0)
G•C→T•A		1 (7.1)		43 (25.0)
A•T→T•A		4 (28.6)		52 (30.2)
A•T→C•G		3 (21.4)		39 (22.7)
Deletions	6 (19.4)		59 (11.5)	
Frameshifts (−1)	1 (3.2)		54 (10.5)	
Frameshifts (+1)	0 (0)		12 (2.3)	
Complexes	2 (6.5)		15 (2.9)	
Tandems	0 (0)		8 (1.6)	
Insertions	0 (0)		5 (1.0)	
Uncharacterized splices[d]	3		144	

[a] We used only RT-PCR data from our analyses. The Curry control group also included only males. We found no significant differences by χ^2 analysis.

[b] Values in parentheses are percentages of the *Total mutants analyzed* (given in the top row of the table).

[c] Values in parentheses are percentages of *Transitions* or *Transversions*. The category values are offset to the right of the main column so they are not added twice into the *Total mutants analyzed*.

[d] These values are not included in the *Total mutants analyzed* in the top row.

Table 39. *HPRT* Mutational Spectra: Several Datasets[a]

Mutation Type	Ma et al 2000[b] Control *n* (%)	Ma et al 2000[b] BD-Exposed *n* (%)	Curry et al 1999[c] Control *n* (%)	Current Study Multiplex-PCR Analysis of Germinal Mutations[d,e] Control *n* (%)	Monomer *n* (%)	Polymer *n* (%)	Total BD-Exposed *n* (%)
Large Deletion Mutations[d]							
Total mutants analyzed	217 (100)	143 (100)	Not given	94 (100)	88 (100)	92 (100)	180 (100)
Deletions[f]	21 (9.7)	25 (17.5)	Not given	14 (14.9)	21 (23.9)	10 (10.9)	31 (17.2)
Total-gene (exons 1–9)	6 (2.8)	3 (2.1)		4 (4.3)	6 (6.8)	3 (3.3)	9 (5.0)
Multiple-exon	14 (6.5)	19 (13.3)		3 (3.2)	9 (10.2)	2 (2.2)	11 (6.1)
Single-exon	1 (0.5)	3 (2.1)		7 (7.4)	6 (6.8)	5 (5.4)	11 (6.1)
Other	196 (90.3)	118 (82.5)	Not given	80 (85.1)	67 (76.1)	82 (89.1)	149 (82.8)
cDNA Sequence Analysis (RT-PCR)[e]							
Total mutants analyzed	90 (100)	85 (100)	657 (100)	35 (100)	35 (100)	28 (100)	63 (100)
Base substitutions	76 (84.4)[g]	58 (68.2)[h]	360 (54.8)	22 (62.9)	20 (57.1)	15 (53.6)	35 (55.6)
Deletions	5 (5.5)	8 (9.4)	59 (9.0)	6 (17.1)	8 (22.9)	6 (21.4)	14 (22.2)
Frameshifts (−1)	2 (2.2)	9 (10.5)	54 (8.2)	1 (2.9)	4 (11.4)	0 (0)	4 (6.3)
Frameshifts (+1)	2 (2.2)	1 (1.2)	12 (1.8)	0 (0)	0 (0)	0 (0)	0 (0)
Other (complex and undefined)	5 (5.5)	9 (10.5)	172 (26.2)	6 (17.1)	3 (8.6)	7 (25.0)	10 (15.9)
Single Base Substitutions[e]							
Total mutants analyzed	75 (100)	56 (100)	360 (100)	22 (100)	20 (100)	15 (100)	35 (100)
Transitions							
G•C→A•T	32 (42.7)	21 (37.5)	141 (39.1)	4 (18.2)	7 (35.0)	5 (33.3)	12 (34.3)
A•T→G•C	13 (17.3)	8 (14.3)	47 (13.1)	4 (18.2)	4 (20.0)	3 (20.0)	7 (20.0)
Transversions							
G•C→C•G	9 (12.0)	5 (8.9)	38 (10.6)	6 (27.3)	3 (15.0)	1 (6.7)	4 (11.4)
G•C→T•A	5 (6.7)	4 (7.1)	43 (11.9)	1 (4.5)	2 (10.0)	2 (13.3)	4 (11.4)
A•T→T•A	6 (8.0)	9 (16.1)	52 (14.4)	4 (18.2)	3 (15.0)	0 (0)	3 (8.6)
A•T→C•G	10 (13.3)	9 (16.1)	39 (10.8)	3 (13.6)	1 (5.0)	4 (26.7)	5 (14.3)
Number at G•C[i]	46 (61.3)	30 (53.4)	222 (61.7)	11 (50.0)	12 (60.0)	8 (53.3)	20 (57.1)
Number at A•T[i]	29 (38.7)	26 (46.4)	138 (38.3)	11 (50.0)	8 (40.0)	7 (46.7)	15 (42.9)

[a] Unless noted otherwise, all values in parentheses are percentages of the *Total mutants analyzed* (given in the first row of each section). Values offset to the right of the main column should not be added twice into the *Total mutants analyzed*.

[b] Subjects were male workers (who do not smoke) in a high-BD-exposure setting in a Texas SBR production facility. The control subjects were employees of the University of Texas Medical Branch matched by age with the exposed subjects.

[c] An article summarizing background mutational spectra in normal humans using data from 795 mutations from 24 published papers.

[d] These results from the current study are also presented in the top portion of Table 37.

[e] These results from the current study are also presented in Table 30.

[f] The following three rows separate the Deletions into three categories. The values in parentheses, however, are percentages of the *Total mutants analyzed*.

[g] Includes one tandem mutation not included in the distribution of single base substitutions below.

[h] Includes two tandem mutations not included in the distribution of single base substitutions below.

[i] The values in these two rows are offset to the right because together they summarize the two categories *Transitions* and *Transversions*, and both the numbers and the percentages are subsets of the *Total mutants analyzed*.

BIOMARKERS OF EFFECT: CYTOGENETIC ANALYSIS

One indicator of human exposure to mutagens and carcinogens in occupational and environmental surroundings is the level or frequency of chromosomal aberrations, which is detected via cytogenetic analysis of PBLs. Increased levels of chromosomal aberrations can also indicate increased risks of cancer occurring later, as has been shown in epidemiologic studies in Scandinavia (Hagmar et al 1998) and Italy (Bonassi 1999). Chromosomal aberrations in somatic cells may activate oncogenes or inactivate tumor suppressor genes. Changes in the number and structure of chromosomes also are associated with the transformation of normal cells into tumor cells. The frequency of chromosomal aberrations may be affected as well by life style factors such as the level of antioxidant vitamins [A, E, and C] in the diet (Šrám et al 1983).

SCEs are the reciprocal exchanges of identical sequences of DNA between two sister chromatids within chromosomes; they may also reflect the interactions of chemicals with DNA. The molecular mechanisms responsible for SCEs, their biological significance, and their relation to chromosomal aberrations are not understood, except that, in contrast to chromosomal aberrations, increased frequencies of SCEs have not been associated with increased risks of cancer (Hagmar et al 1998; Bonassi et al 1999).

Several in vitro and in vivo studies have investigated the effects of BD and its metabolites on cytogenetic endpoints in rodents and in humans (Pacherrotti et al 1998; see also the earlier Overview section). BD has been shown to increase the frequencies of SCEs in mouse but not rat bone marrow. Similarly, only mice have shown other cytogenetic changes following in vivo exposure to BD; nevertheless, increased micronucleus frequencies in peripheral blood erythrocytes, splenocytes, or both, and chromosomal rearrangements (but not aneuploidy, which results from the loss or gain of whole chromosomes) in bone marrow have been observed in both mice and rats after in vivo exposure to BD metabolites. Of special interest is the effect of BD on gametes; dominant lethal mutations, heritable translocations, and sperm-head abnormalities have been observed only in mice after exposure to BD (reviewed in IARC 1999). Therefore, chromosome-level changes have frequently been observed in vivo in mouse somatic or germ cells after BD exposure, although such changes have not been seen in rats. Exposure to BD metabolites, however, have produced cytogenetic effects in both species (Pacherrotti et al 1998).

In human cells, direct in vitro applications of the metabolites BDO and BDO_2 have induced SCEs in lymphocytes; in this, BDO_2 has been more potent than BDO (Sasiadek et al 1991a). Certain metabolic genotypes in humans influence susceptibility to SCE induction by BD metabolites. In vitro, lymphocytes from individuals with the *GSTM1*-null genotype were more susceptible to SCE induction by BDO, whereas lymphocytes from individuals with the *GSTT1*-null genotype were more susceptible to SCE induction by BDO_2 (Norppa et al 1995, Uuskula et al 1995). The *GST* genotype did not influence SCE induction by BDO-diol (Bernardini et al 1996). Aneuploidy has also been reported in human lymphocytes in vitro after exposure to either BDO or BDO_2, with BDO_2 again being more potent; in one report, the aneuploidy appeared to be specific to chromosomes 12 and X (Xi et al 1997).

Although these in vitro findings clearly demonstrate that BD metabolites are genotoxic to human cells, they do not indicate what may occur in vivo after BD exposure when the parent compound must be metabolized into its genotoxic intermediates. This can be determined only by direct studies of humans exposed to BD.

The chromosomal effects of BD have been evaluated in studies of exposed workers, but results have been inconsistent. Positive results from in vivo studies include an increase of chromosomal aberrations and SCEs in BD-exposed Czech workers sampled in 1994 (Šrám et al 1998) and abnormal DNA repair activities in cells from BD-exposed workers in Texas (Hallberg et al 1997). One study, in which cells from BD-exposed workers in Texas were challenged in vitro with a clastogenic agent, showed increased chromosomal breakage compared with cells from control subjects (Au et al 1995). The results of that study might be interpreted as demonstrating chromosome-level damage in vivo that required an external stress to convert the damage to chromosomal breakage.

The same Czech worker group that had shown increases in chromosomal aberrations when sampled in 1994 had also been examined in 1992 and 1993; at that time, Sorsa and associates (1994) found no evidence of increases in chromosomal aberrations, SCEs, or micronuclei. However, when these same cytogenetic data were reanalyzed with respect to genotypes, the same investigators found that BD-exposed workers with the *GSTT1*-null genotype had significantly higher frequencies of chromosomal aberration than the appropriate control group (Sorsa et al 1996). The *GSTM1* genotype conferred no similar susceptibility. A study by Hayes and associates (2000) of Chinese workers exposed to BD analyzed SCEs and aneuploidy and found no effects at exposure levels comparable to those reported in other studies (Sorsa et al 1994, 1996).

The current project studied a group of workers from the same petrochemical company in the Czech Republic that

had been sampled in 1992, 1993, and 1994. The samples used for the 1992 and 1993 analyses were obtained at the beginning of October, whereas the samples for the 1994 analysis were acquired at the end of November. The timing of samples is important because most workers are on vacation for 3 to 4 weeks in August; thus, samples acquired in October may show the effects of this period of nonexposure, whereas samples acquired in November would reflect approximately 3 full months of exposure. The exposed subjects for the 1992 study consisted of workers in both the monomer production and the polymerization units, whereas the 1993 and 1994 samples were collected exclusively from workers in the monomer production unit. The control subjects for all 3 years were power plant workers.

During our analysis of the 1994 samples, we also analyzed the 1993 samples for the occurrence of high frequency cells (HFCs). These are recorded as the percentage of cells (lymphocytes) in which a high frequency of SCEs per cell are found. HFCs may sensitively detect chemical exposure (Carrano and Moore 1982). Multifactorial analysis of all results from the 1992, 1993, and 1994 samples showed that chromosomal aberrations and HFCs were the predominant effects of BD exposure, although these were significantly increased only in the 1994 samples. Furthermore, no effects were observed in any of the samples when assessed using the comet or micronucleus assays (Tates et al 1996, Šrám et al 1998). For these reasons, only chromosomal aberrations and SCEs were selected for cytogenetic analysis in the current study.

METHODS

Chromosomal Aberrations Identified by the Conventional Method

We set aside fresh, whole-blood samples (5–10 mL per subject) before we fractionated and cryopreserved samples for studies in other laboratories. The whole-blood samples were coded only with each subject's three-digit identification number.

We established cultures within 24 hours after blood was collected, according to the methods described by Sorsa and associates (1994). Whole-blood lymphocyte cultures were set up in tissue culture flasks or in penicillin flasks; each flask contained 0.6 mL whole blood and 7.5 mL cultivation medium (which, for one flask, included 1.06 mL RPMI 1640 [SEVAC, Prague], 1.80 mL BCS [OPAVAC, Opava], 4.24 mL distilled water, 0.10 mL L-glutamine, 0.16 mL $NaHCO_3$, and 0.10 mL PHA [HA-15, Murex, UK]). The cultures for chromosomal aberrations were cultivated at 37°C for 48 hours; 2 hours before the end of cultivation, colchicine (Fluka) was added in a final concentration of 0.5 μg/mL culture. The cells were then treated with a hypotonic solution of KCl (0.075 M) for 10 minutes and fixed three times with methanol:acetic acid (3:1). The slides were dried for 24 hours, stained with a 5% solution of Giemsa-Romanowski, and coded with the subject's three-digit identification number.

For each subject, 100 metaphases were analyzed at a magnification of ×1000 under immersion. We scored four forms of chromosomal aberrations: breaks and exchanges in chromatids and chromosomes. Aberrant cells were defined as cells bearing chromosomal breaks and exchanges; gaps in chromosomes were not counted as aberrant cells. The number of chromosomal breaks/cell were also counted (a chromosomal break was counted as 1, an exchange was counted as 2 breaks).

Chromosomal Changes Identified by FISH

Stable chromosomal translocations reflect true in vivo events that result in genetic effects; the frequency with which they occur may be significantly affected by the age of the subject. The FISH method identifies how exposure to a mutagen affects the frequency of stable chromosomal translocations. It uses painting probes to label individual chromosomes. (Until now, it has mostly been used to analyze the effects of different types of radiation on chromosomes.) With the Protocol for Aberration Identification and Nomenclature Terminology (PAINT), it is possible to classify exactly each type of chromosomal rearrangement (Tucker et al 1995):

- *Chromosomal aberration*. A generic term meaning a gross change in chromosomal structure.
- *Translocation*. The movement of a part of a chromosome to another chromosome or to a different location in the same chromosome (a change in the position of a segment).
- *Reciprocal translocation*. The exchange of parts between two chromosomes whereby each receives part of the other in place of the part that it lost. (This is a prototypic "stable translocation" in that no genetic material is lost to the cell so it does not die.)
- *Insertion*. The translocation of a piece of a chromosome between two breaks; the piece may come from the same or another chromosome.
- *Conjunction.* A connection or link among colors between chromosomes.

Whole blood (1 mL) was added to 10 mL culture medium RPMI 1640 (SEVAC, Prague) that was supplemented with 20% fetal BCS, phytohemagglutinin (0.10 mL per total volume [7.5 mL medium + 0.6 mL blood]), and L-glutamine (0.5 mg/mL). To obtain a sufficient number of

mitoses, the cultures for analysis by FISH were harvested after 48 or 68 hours of incubation; at 1 hour before the end of the incubation, colchicine (Fluka) was added to a final concentration of 0.5 μg/mL. Slides were prepared using the air-dry method (Verma and Babu 1989) and stored at −20°C.

FISH analysis using commercial whole-chromosome painting probes in different colors (Biovation, Aberdeen, UK) for chromosomes 1 and 4 was carried out according to the manufacturer's chromosome painting protocol. After incubating the slides with the probes, slides were mounted in a 4,6-diamidino-2-phenylindole (DAPI) antifade solution and examined using an Olympus BX 60 fluorescence microscope equipped with a triple-band pass filter for DAPI and the fluorochrome tracers (fluorescein isothiocyanate and cyanogen 3) (Rubeš et al 1998). We analyzed 500 metaphases per subject.

All aberrant cells were classified according to PAINT (Tucker et al 1995). The protocol defines translocations and insertions as follows: A translocation is a rearranged chromosome with a single centromere and is to be counted as an aberration; the translocated chromosome must exhibit at least two colors. An insertion is the presence of acentric chromosomal material situated within another chromosome; the material flanking either side of an insertion must be of the same color.

Genomic frequencies of stable translocations per 100 cells (F_G/100) were calculated according to Lucas and associates (1993) using the equation:

$$F_G/100 = F_{gr}/2.05\,[f_r\,(1 - f_r) + f_g\,(1 - f_g) - f_r f_g],$$

where F_{gr} is the translocation frequency measured by FISH after two-color painting, and f_r and f_g are the fractions of the genome painted red and green, respectively.

SCEs

Cultures were established within 24 hours after blood was collected according to the method described by Sorsa and associates (1994). Whole-blood lymphocyte cultures were set up in tissue culture flasks or in penicillin flasks. Each culture contained 0.6 mL whole blood, 7.5 mL cultivation medium (which, for one flask, included 1.06 mL RPMI 1640 [SEVAC], 1.80 mL BCS [OPAVAC], 4.24 mL distilled water, 0.1 mL L-glutamine, 0.16 mL $NaHCO_3$, and 0.1 mL PHA [HA-15, Murex, UK]), and 5-bromo-2′-deoxyuridine (in a final concentration of 10 μg/mL culture). The cultures for SCE were cultivated for 68 hours; 2 hours before the end of cultivation, colchicine was added at a final concentration of 0.5 μg/mL culture. The cells were treated with a hypotonic solution of KCl (0.075 M) for 10 minutes and fixed three times with methanol:acetic acid (3:1). Cells were then added to microscope slides. After several days, slides were placed into Hoechst H 33258 working solution, exposed to UV light for 1.5 hours, incubated in a water bath at 60°C for 2 hours, and then stained with a 5% solution of Giemsa-Romanowski.

For SCE scoring, 50 second-division metaphases per subject were analyzed according to the method of Perry and Wolf (1974). Cells were analyzed at a magnification of ×1000 under immersion. Results were expressed as the number of SCEs/cell. The percentage of HFCs (defined as more than 12 SCEs per cell) was calculated according to Carrano and Moore (1982).

Statistical Methods

A complete description of all statistical methods is found in the earlier section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Data Processing and Statistical Analysis.

RESULTS

Cytogenetic studies included assays for (1) chromosomal aberrations identified by the conventional method (aberrant cells and breaks/cell); (2) chromosomal changes identified by FISH (chromosomal aberrations, translocations, reciprocal translocations, conjunctions, and F_G/100); and (3) SCEs (SCEs/cell and percentage of HFCs). Chromosomal aberrations and translocations involve gross changes in the genetic material. SCEs measure reciprocal exchanges between sister chromatids without the apparent loss of genetic material. They are sensitive biomarkers of exposure for many chemicals, but do not have a genotoxic consequence. Individual results for each subject from all three exposure groups are presented in Table 40 for several cytogenetic endpoints.

Chromosomal Aberrations Identified by the Conventional Method

Successful analyses of chromosomal aberrations were accomplished for 82 (99%) of the subjects. Only one sample, from a monomer production worker, was lost to analysis. Table 41 presents the group mean percentage of aberrant cells and the mean number of breaks/cell for the three exposure groups. None of these values differed significantly between pairs of groups. Also shown in Table 41 are cumulative results from our laboratory for aberrant cells from normal control individuals in the Czech Republic. Although this value is provided for reference, the group represents normal blood-bank volunteers from throughout the nation and may not be strictly comparable to the administrative control group in the current study.

Table 40. Individual Data from Cytogenetic Analyses for Each Subject

Subject	Age	Exposure[a] (mg/m³)	Smoking Status[b]	Genotype[c]		Aberrant Cells (%)	Breaks/ Cell	F_G/ 100[d]	SCEs/ Cell	% HFC
				GSTM1	*GSTT1*					
Administrative Control Subjects (n = 25)										
108	66	0.050 (0.009–0.090)	NS	–	+	2	0.02	3.51	4.78	0
109	61	0.029 (0.009–0.048)	S	+	+	4	0.05	1.12	7.16	6
110	58	0.010 (0.007–0.013)	—[e]	–	+	4	0.05	5.17	5.04	0
114	55	0.083 (0.040–0.125)	NS	–	+	1	0.01	0.81	6.26	0
115	52	0.013 (0.002–0.024)	S	–	–	1	0.01	3.60	6.52	2
116[f]	50	0.023 (0.018–0.029)	NS	+	+	4	0.05	2.55	5.82	4
117[f]	49	0.023 (0.018–0.029)	NS	+	+	1	0.01	2.69	5.84	0
118	48	0.015 (0.015–0.015)	NS	+	–	1	0.01	2.62	6.72	4
119	48	0.059 (0.059–0.059)	S	+	+	3	0.03	1.27	5.40	0
120	45	0.026 (0.026–0.026)	NS	+	+	0		1.41	5.82	0
121	42	0.002 (0.002–0.002)	S	–	+	0		2.23	8.28	22
122	42	0.022 (0.022–0.022)	S	+	–	1	0.02	7.20	6.60	0
123	40	0.002 (0.002–0.002)	S	–	+	2	0.03	4.13	7.02	2
155	33	0.068 (0.068–0.068)	NS	+	+	2	0.02	3.43	6.94	6
156	35	0.002 (0.002–0.002)	NS	+	+	1	0.02	2.26	4.66	0
166	33	0.002 (0.002–0.002)	S	–	+	0		9.24	7.50	0
167	32	0.002 (0.002–0.002)	NS	–	–	2	0.02	0.37	6.50	2
168	29	0.002 (0.002–0.002)	NS	+	+	1	0.01	0.71	6.78	8
169	28	0.031 (0.031–0.031)	NS	+	+	2	0.02	1.38	6.14	0
170	28	0.044 (0.044–0.044)	NS	+	+	1	0.01	1.68	7.26	4
171	20	0.042 (0.042–0.042)	NS	+	+	3	0.03	5.26	5.68	0
172	26	0.013 (0.013–0.013)	NS	+	+	0		2.67	6.68	4
182	24	0.013 (0.013–0.013)	S	+	+	1	0.01	1.76	6.84	2
183	21	0.002 (0.002–0.002)	NS	–	–	1	0.01	2.07	6.12	0
184	22	0.009 (0.009–0.009)	S	+	+	1	0.02	NA[g]	5.16	2
Monomer Production Workers (n = 24)										
102	49	0.062 (0.002–0.257)	NS	+	+	0		9.62	4.94	0
103	48	0.398 (0.002–1.252)	S	+	+	2	0.11	1.86	7.48	4
104	20	0.093 (0.004–0.332)	NS	+	–	0		0.72	5.18	0
105	20	0.674 (0.040–2.803)	S	–	+	3	0.03	0.54	6.86	0
113	54	0.243 (0.002–0.779)	S	+	+	3	0.04	2.67	7.30	12
124	52	0.161 (0.002–0.860)	NS	–	+	0		2.55	NA	
125	47	0.195 (0.002–1.058)	S	+	+	1	0.01	8.29	6.46	2
126	47	0.238 (0.013–1.030)	S	–	–	NA		4.14	6.70	2
127	52	0.396 (0.018–3.388)	NS	–	–	4	0.04	2.32	NA	
128	25	0.411 (0.002–2.059)	S	+	+	2	0.03	0.46	NA	
140	53	0.053 (0.002–0.246)	S	+	+	3	0.04	NA	5.02	0
141	53	0.146 (0.002–1.135)	S	–	+	2	0.02	0.89	NA	
142	28	0.694 (0.011–3.991)	S	+	+	1	0.01	1.02	NA	
143	39	0.689 (0.002–2.556)	S	–	+	2	0.03	2.29	6.50	6
144	22	1.861 (0.035–5.366)	S	–	+	2	0.02	1.37	6.68	8
147	51	0.072 (0.002–0.315)	NS	–	+	1	0.01	4.17	NA	

(Table continues next page)

[a] Mean value with range given in parentheses.

[b] S = smoker, NS = nonsmoker.

[c] + = carries this gene; – = does not carry this gene (null genotype).

[d] Frequency per 100 cells.

[e] This subject did not provide information about smoking status.

[f] Exposure measurements were not obtained for these subjects and they were assigned the mean values for the group.

[g] NA = data from these subjects were not analyzed.

Table 40 (*Continued*). Individual Data from Cytogenetic Analyses for Each Subject

Subject	Age	Exposure[a] (mg/m³)	Smoking Status[b]	Genotype[c] GSTM1	Genotype[c] GSTT1	Aberrant Cells (%)	Breaks/ Cell	F_G/ 100[d]	SCEs/ Cell	% HFC
Monomer Production Workers (n = 24) **(*Continued*)**										
149	42	0.174 (0.002–0.838)	NS	+	+	2	0.02	1.37	4.33	0
150	31	0.165 (0.002–0.585)	NS	+	–	1	0.01	1.36	7.26	8
151	50	1.266 (0.011–2.970)	S	–	+	2	0.02	0.62	5.34	0
152	50	3.516 (0.029–19.910)	NS	+	+	0		0.84	5.34	0
157	48	0.416 (0.002–1.956)	NS	–	+	1	0.01	2.73	6.54	0
177	21	0.607 (0.029–1.896)	S	+	+	0		0.53	6.20	2
178	21	2.530 (0.002–19.532)	NS	+	+	3	0.03	0.87	6.22	2
181	52	0.348 (0.002–2.851)	NS	+	+	0		2.77	NA	
Polymerization Workers (n = 34)										
101	51	2.421 (0.002–6.765)	S	+	+	1	0.01	3.98	6.70	6
106	25	1.503 (0.037–10.619)	NS	–	–	1	0.01	1.17	5.42	0
107	21	1.119 (0.68–4.415)	NS	+	+	0		2.99	4.50	0
111	49	0.838 (0.029–3.247)	S	+	+	1	0.01	5.17	NA	
112	54	0.641 (0.002–1.762)	S	+	+	3	0.03	3.68	7.04	0
129	45	7.408 (0.062–39.030)	S	–	+	4	0.05	2.78	6.86	8
130	53	0.188 (0.011–0.689)	S	+	+	1	0.01	2.67	NA	
131	52	0.470 (0.002–2.317)	S	–	+	3	0.05	3.57	9.02	16
132	33	0.326 (0.002–2.317)	S	–	+	1	0.02	0	6.18	2
133	27	1.154 (0.011–4.715)	NS	+	+	2	0.02	1.49	5.22	2
134	34	0.286 (0.002–1.177)	NS	–	+	0		3.61	5.76	0
135	51	1.045 (0.002–3.155)	S	+	+	2	0.02	4.64	6.88	8
136	41	0.657 (0.011–2.893)	S	–	+	1	0.01	3.28	5.72	4
137	58	0.144 (0.002–0.587)	NS	+	+	1	0.01	NA	6.34	2
138	46	0.262 (0.002–1.208)	NS	–	+	0		NA	6.56	6
139	41	1.076 (0.002–7.341)	NS	+	–	2	0.02	3.14	5.98	2
145	42	0.251 (0.020–1.740)	NS	+	+	1.5	0.02	4.31	6.36	2
146	25	1.211 (0.031–5.243)	S	+	+	3	0.03	1.83	6.42	6
148	42	0.336 (0.011–0.726)	S	–	+	2	0.03	1.81	6.03	3
153	24	1.754 (0.002–9.530)	S	+	+	1	0.01	1.84	8.04	10
154	51	1.903 (0.002–6.613)	NS	+	+	2	0.02	2.90	5.52	0
158	52	0.232 (0.002–0.801)	NS	+	+	3	0.04	7.67	6.16	0
159	50	0.990 (0.167–4.343)	NS	–	–	1	0.02	4.92	6.54	0
160	22	3.650 (0.002–19.362)	S	–	–	1	0.01	NA	6.52	6
161	51	1.206 (0.018–2.570)	S	+	+	1	0.01	1.25	7.42	6
162	27	2.335 (0.002–18.718)	NS	+	+	3	0.03	0.46	6.16	2
164	21	9.244 (0.029–37.376)	NS	+	+	1	0.01	0.90	7.02	6
165	27	0.040 (0.011–0.117)	NS	+	+	2	0.02	4.81	6.98	2
173	28	0.173 (0.002–0.405)	NS	+	+	1	0.01	2.09	7.06	2
174	37	2.088 (0.002–6.365)	NS	+	+	0		2.53	6.32	2
175	22	4.669 (0.002–19.659)	S	+	+	2	0.03	0	6.16	0
176	32	4.733 (0.002–27.562)	NS	–	–	3	0.04	7.87	NA	
179	51	3.792 (0.002–28.228)	S	+	+	0		4.59	7.70	6
180	50	2.848 (0.110–18.229)	NS	+	+	2	0.02	4.13	6.44	2

[a] Mean value with range given in parentheses.

[b] S = smoker, NS = nonsmoker.

[c] + = carries this gene; − = does not carry this gene (null genotype).

[d] Frequency per 100 cells.

[e] This subject did not provide information about smoking status.

[f] Exposure measurements were not obtained for these subjects and they were assigned the mean values for the group.

[g] NA = data from these subjects were not analyzed.

Table 41. Chromosomal Aberrations Identified by the Conventional Method: Effect of BD Exposure by Group

	Control (n = 25)	Monomer (n = 23)[a]	Polymer (n = 34)	National Control Group[b] (n = 1156)
BD exposure[c] (mg/m[3])	0.023 (0.002–0.083)	0.642 (0.053–3.516)	1.794 (0.040–9.244)	—
Aberrant cells (%)[d]	1.56 ± 1.23	1.52 ± 1.20	1.54 ± 1.05	1.04 ± 0.86
Breaks/cell[d]	0.018 ± 0.015	0.019 ± 0.017	0.018 ± 0.014	—

[a] Samples from one subject were lost to analysis.

[b] Control data from individuals in the Czech Republic.

[c] Group mean exposure with range of individual subjects' mean exposure levels in parentheses.

[d] Mean ± SD.

Table 42. Chromosomal Aberrations Identified by the Conventional Method: Effect of Genotype[a]

	Control (n = 25)	Monomer (n = 21)	Polymer (n = 30)	All Subjects (n = 76)
ADH2[b]				
Aberrant cells (%)				
Arg47/Arg47	1.35 ± 1.03	1.56 ± 1.20	1.45 ± 0.99	1.44 ± 1.05
Arg47/His47	4.00 ± 0.00	1.67 ± 1.53	3.50 ± 0.71	2.86 ± 1.47
Breaks/cell				
Arg47/Arg47	0.016 ± 0.012	0.019 ± 0.017	0.017 ± 0.013	0.017 ± 0.014
Arg47/His47	0.050 ± 0.000	0.020 ± 0.020	0.040 ± 0.014	0.034 ± 0.019
EH113[c]				
Aberrant cells (%)				
Tyr113/Tyr113	1.73 ± 1.28	1.00 ± 0.71	1.33 ± 1.11	1.46 ± 1.15
Tyr113/His113	1.33 ± 1.37	1.83 ± 1.19	2.17 ± 1.00	1.83 ± 1.17
His113/His113	1.25 ± 0.96	1.50 ± 1.73	1.33 ± 1.03	1.36 ± 1.15
Breaks/cell				
Tyr113/Tyr113	0.021 ± 0.015	0.010 ± 0.007	0.015 ± 0.014	0.017 ± 0.014
Tyr113/His113	0.017 ± 0.018	0.024 ± 0.018	0.026 ± 0.015	0.023 ± 0.016
His113/His113	0.013 ± 0.010	0.018 ± 0.021	0.015 ± 0.010	0.015 ± 0.013
EH139[c]				
Aberrant cells (%)				
Arg139/Arg139	1.50 ± 0.71	3.00 ± 1.00	2.00[d]	2.33 ± 1.03
Arg139/His139	1.73 ± 1.35	0.00 ± 0.00	1.20 ± 0.92	1.35 ± 1.19
His139/His139	1.42 ± 1.24	1.50 ± 1.03	1.76 ± 1.18	1.59 ± 1.13
Breaks/cell				
Arg139/Arg139	0.020 ± 0.000	0.033 ± 0.006	0.020[d]	0.027 ± 0.008
Arg139/His139	0.021 ± 0.016	0.000 ± 0.000	0.015 ± 0.014	0.017 ± 0.016
His139/His139	0.016 ± 0.015	0.019 ± 0.017	0.020 ± 0.014	0.019 ± 0.015

[a] All values are presented as means ± SD.

[b] The overall difference between genotypes was significant at $P < 0.001$ by ANOVA.

[c] The mean differences were not significant.

[d] Means with no SDs indicate that only one subject had this genotype.

Both the percentage of aberrant cells and the breaks/cell were associated with the metabolic genotypes determined for these subjects (Table 42). The most consistent associations were with the *ADH2* polymorphisms, which were associated with differences in both of these endpoints. Subjects with the *Arg47*/*Arg47* homozygous genotype at this locus had significantly lower mean values for percentage of aberrant cells and breaks/cell than did the subjects with the *Arg47*/*His47* heterozygous genotype (no subjects with *His47*/*His47* homozygous genotype were included in the study). Regression analyses, however, provided no evidence that these genotypic differences were related to BD exposures.

Although the *EH* polymorphisms were related to no statistically significant differences in the percentage of aberrant cells or breaks/cell and to no significant exposure-group–by–genotype interactions (Table 42), regression analyses of the cytogenetic endpoints on ln(BD-exposure-level–by–genotype) did reveal some statistically significant associations. At the *EH113* locus, workers with the *Tyr113*/*Tyr113* homozygous genotype had lower slopes for percentage of aberrant cells and breaks/cell than workers with either the *His113*/*His113* homozygous or the *His113*/*Tyr113* heterozygous genotype, but the slopes differed significantly only from those for the *His113*/*Tyr113* genotype (Figure 27). The *Tyr113* allele, which is associated with lower production of these cytogenetic changes when the individual is homozygous for it, is the allele with higher activity at the locus.

At the *EH139* locus (Figure 28), workers with the *Arg139*/*Arg139* homozygous genotype had a statistically greater slope for percentage of aberrant cells than did workers with either the *His139*/*His139* homozygous or the *Arg139*/*His139* heterozygous genotype. The lower production of aberrant cells was associated with either homozygosity or heterozygosity for the lower-activity allele at the *EH139* locus, which, in this case, is the variant *His139* allele. Overall, these results comparing the *EH* genotypes are conflicting. The *EH* genotypes at the *113* locus associated with the higher enzyme activity showed lower genotoxicity as expected; but the reverse was seen for the *EH* genotypes at the *139* locus.

No significant associations were observed between either percentage of aberrant cells or breaks/cell and *CYP2E1*, *ADH3*, *GSTM1*, or *GSTT1* genotypes (data not shown). The lack of association with the *GST* polymorphisms is in contrast to other studies (eg, Sorsa et al 1996).

Analyses of the relation between smoking and the percentage of aberrant cells or breaks/cell showed no effect of smoking on the percentage of aberrant cells (Figure 29, top panel); however, the mean number of breaks/cell was significantly higher in smokers than nonsmokers (Figure 29, bottom panel), both overall ($P = 0.031$ by Mann-Whitney test) and within the monomer group ($P = 0.022$ by Mann-Whitney test). Neither of these two endpoints was significantly correlated with average BD exposure, regardless of whether the control group was included or excluded from the analysis. Regression analyses to adjust for age and current

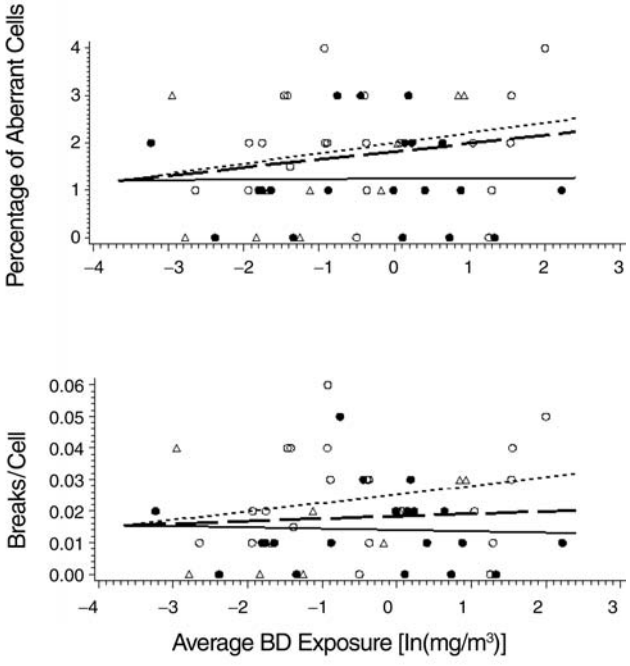

Figure 27. Percentage of aberrant cells (top) or number of breaks per cell (bottom) vs average BD exposure by *EH113* genotypes. ● and solid line indicate *Tyr113/Tyr113* genotype; ○ and short-dashes line indicate *Tyr113/His113* genotype; △ and long-dashes line indicate *His113/His113* genotype. Slope for *Tyr113/Tyr113* differs significantly from slope for *Tyr113/His113* (top: $P = 0.040$; bottom: $P = 0.020$).

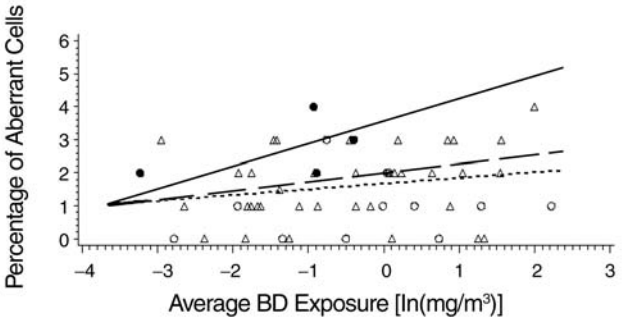

Figure 28. Percentage of aberrant cells vs average BD exposure by *EH139* genotypes. ● and solid line indicate *Arg139/Arg139* genotype; ○ and short-dashes line indicate *Arg139/His139* genotype; △ and long-dashes line indicate *His139/His139* genotype. Slope for *Arg139/Arg139* differs significantly from slopes for *Arg139/His139* ($P = 0.004$) and *His139/His139* ($P = 0.029$).

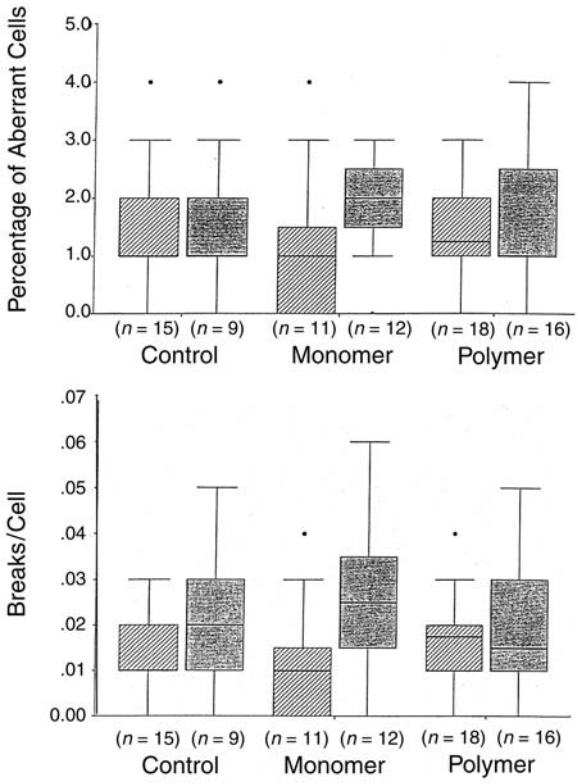

Figure 29. Summary of the data comparing smoking status with SCEs and HFCs. Hatched boxes = subjects who do not currently smoke; gray boxes = subjects who do. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. Error bars are the highest and lowest values within 1.5 box-lengths from each percentile. The line within each box marks the position of the median value. The dots are values that are more than 1.5 box-lengths from the 75th percentile.

smoking status also revealed no significant relation between either aberrant cells or breaks/cell and average BD exposure.

On the basis of information supplied on the questionnaires, we analyzed several small groups of subjects with particular characteristics (regardless of exposure group), comparing each small group with the remainder of the subjects. The percentages of aberrant cells and breaks/cell were both significantly related to

- a history of hepatitis (in 6 subjects),
- a vaccination in the preceding 12 months (in 14 subjects), and
- the use of herbicides or pesticides during the preceding 3 months (in 13 subjects).

For each of these two endpoints, the values were significantly higher among workers having the characteristic than workers without the characteristic.

In summary, no significant relations could be identified between group mean chromosomal aberrations determined by the conventional method and BD exposure levels. Associations with smoking, some genotypes, and several questionnaire variables were observed. Some genotype associations were conflicting. Specifically, regression analyses showed that BD exposure was related to the production of fewer chromosomal aberrations in the *EH* genotypes with both the higher-activity and lower-activity alleles, depending on the *EH* locus, but no relation was found between BD exposure and aberrations associated with the *GST* genotypes.

Chromosomal Changes Identified by FISH

The chromosomal changes identified by FISH included chromosomal aberrations, translocations, reciprocal translocations, and conjunctions (all reported per 1000 metaphases), plus F_G/100. Successful analysis by FISH was accomplished for 78 (94%) of the subjects: One sample (4%) was lost from an administrative control subject, one (4%) from a monomer production worker, and three (9%) from polymerization workers. The group means for the frequencies of these chromosomal changes are given in Table 43. None of the mean differences between groups were statistically significant for these cytogenetic endpoints.

All group mean values for each chromosomal change determined by FISH were significantly lower in the two subjects with the *CYP2E1 C1/C1* homozygous genotype than in the 70 subjects with the *C1/C2* heterozygous genotype (Table 44). In addition, we noted a significant exposure-group–by–genotype interaction between the *EH113* polymorphisms for F_G/100 ($P = 0.034$). Subjects in the control and polymer groups identified with the *Tyr113/His113* heterozygous genotype at this locus showed *higher* F_G/100 than did subjects in the same exposure groups who had been identified with either homozygous genotype at this locus; in contrast, subjects in the monomer group identified with this heterozygous genotype showed *lower* F_G/100 than subjects in the same group identified with either homozygous genotype (Table 45). Very similar interactions were also observed for chromosomal aberrations ($P = 0.036$), translocations ($P = 0.034$), and conjunctions ($P = 0.047$), as might be expected because of the high correlations among FISH endpoints.

None of the endpoints examined by FISH revealed any differences between current smokers and nonsmokers. All five FISH endpoints, however, were positively and significantly correlated with age (data not shown). None of the five endpoints was significantly correlated with average BD exposure when all data were included in the analysis.

Table 43. Chromosomal Changes Identified by FISH[a]

Aberration	Control	Monomer	Polymer
All Subjects[b]	(n = 24)	(n = 23)	(n = 31)
Chromosomal aberrations (‰)	4.63 ± 3.27	4.05 ± 3.14	5.48 ± 3.21
Translocations (‰)	7.14 ± 5.71	6.29 ± 6.32	8.30 ± 5.13
Reciprocal translocations (‰)	2.10 ± 2.47	2.38 ± 3.25	2.67 ± 1.94
Conjunctions (‰)	7.95 ± 6.66	6.79 ± 6.21	9.36 ± 6.65
F_G/100	2.67 ± 2.13	2.35 ± 2.36	3.10 ± 1.92
Subjects with Possible Clonal Expansion Excluded	(n = 19)	(n = 21)	(n = 28)
F_G/100	2.06 ± 1.31	1.72 ± 1.14	2.73 ± 1.51[c]

[a] The FISH analyses noted as (‰) are presented per 1000 metaphases. Values are presented as means ± SD.

[b] Subjects lost to analysis: 1 administrative control subject, 1 monomer production worker, and 3 polymerization workers.

[c] Significantly different from monomer group at $P < 0.05$ by Kruskal-Wallis test.

However, when we excluded data from the administrative control subjects, we observed a significant negative correlation between average BD exposure and chromosomal aberrations (data not shown; $r = -0.286$, $P = 0.038$). We then performed regression analyses with each of the FISH endpoints as dependent variables; we used age, current smoking, and average BD exposure as independent variables. These analyses showed no significant effect of BD exposure after we adjusted for age and smoking.

On the basis of information supplied on the questionnaires, we analyzed the following small groups and obtained these results:

- Reciprocal translocations were correlated with cumulative cigarette consumption ($r = 0.229$, $P = 0.047$), were significantly higher in the group of five workers who had a history of anemia ($P = 0.022$), and were significantly lower in four workers with a history of mononucleosis ($P = 0.037$); none of the other FISH endpoints was significantly related to these variables.
- Chromosomal aberrations ($r = -0.272$, $P = 0.019$), translocations ($r = -0.236$, $P = 0.043$), and F_G/100 ($r = -0.236$, $P = 0.043$) were inversely correlated with hours per day of passive exposure to cigarette smoke.
- The mean values of F_G/100 and translocations ($P = 0.029$ for both) were significantly lower in six workers who reported exposure to lead in the previous 3 months.

When we compared the control group in this study with cumulative results from healthy blood donors in the Czech Republic (Rubeš et al 1998), we found that F_G/100 was significantly higher in the current control group than in the group of healthy blood donors. F_G/100 is known to be

Table 44. Chromosomal Changes Identified by FISH: Effects in the *CYP2E1* 5′ Promoter Region Genotype[a]

Aberration	*C1*/*C1* (n = 70)	*C1*/*C2*[b] (n = 2)
Chromosomal aberrations (‰)	4.68 ± 3.20	10.27 ± 3.70
Translocations (‰)	7.19 ± 5.49	18.41 ± 10.40
Reciprocal translocations (‰)	2.32 ± 2.30	8.57 ± 6.10
Conjunctions (‰)	8.01 ± 6.45	19.26 ± 9.20
F_G/100	2.68 ± 2.05	6.88 ± 3.88

[a] The FISH analyses noted as (‰) are presented per 1000 metaphases. Values are presented as means ± SD.

[b] All values are significantly different from the *C1*/*C1* genotype at $P < 0.05$ by t test.

Table 45. F_G/100 Identified by FISH: Effects in the *EH113* Genotype[a,b]

	Control (n = 24)	Monomer (n = 21)	Polymer (n = 27)
Tyr113/*Tyr113*	2.50 ± 1.43	2.98 ± 2.93	2.96 ± 1.35
Tyr113/*His113*	4.03 ± 3.37	1.58 ± 1.12	4.27 ± 2.78
His113/*His113*	1.21 ± 0.58	4.35 ± 4.64	2.76 ± 2.14

[a] Values are shown as means ± SD of stable translocations/100 cells.

[b] We noted a significant exposure-group–by–genotype interaction, which reflects that the *Tyr113*/*His113* genotype had the highest means for the control and polymer groups and the lowest mean for the monomer group.

correlated with age and it can be influenced by clonal amplification of the cells with translocations. For these reasons, we reviewed the FISH data for $F_G/100$; of the cells with a stable translocation, we eliminated those with evidence of possible clonal expansion and reanalyzed the data from all remaining subjects. Table 43 shows the resulting values; the mean difference between the polymer and monomer groups was statistically significant ($P < 0.05$) in this highly restricted analysis, but the biological significance of this finding is uncertain because we found no differences between exposed and control subjects.

SCEs

Assays for SCEs were successfully completed for 73 subjects (88%): None (0%) of the assays were unsuccessful for the administrative control subjects, seven (29%) were unsuccessful for monomer production workers, and three (9%) were unsuccessful for polymerization workers.

Table 46 shows the group mean results for SCEs and the percentage of HFCs; none of these values differed significantly from each other. Furthermore, none of the genotypes determined in this study, including the *GST* polymorphisms, showed an effect on SCEs.

Spearman correlation coefficients were computed to assess relations between SCEs or percentage of HFCs and BD exposures and showed no significant correlations. Analyzing the relation between smoking and mean SCEs or percentage of HFCs revealed significantly higher values for both of these endpoints in smokers compared with nonsmokers, both for the entire study group and within the polymer group. (See Figure 30 for a visual summary that compares data for smoking status with SCEs and percentage of HFCs.) No significant interaction was identified between smoking and exposure group for either endpoint.

Regression analyses showed no significant effect of BD exposure (using either direct exposure measurements or biomarkers as surrogate measures of exposure) on SCEs or percentage of HFCs after adjusting for age and current smoking status. Furthermore, the effect of smoking on these two endpoints remained significant after adjusting for age and BD exposure.

On the basis of information supplied on the questionnaires, we analyzed the following small groups and obtained these results:

- SCEs and percentage of HFCs were related to cumulative cigarette consumption ($r = 0.376$, $P < 0.001$ for SCEs; $r = 0.268$, $P = 0.020$ for HFCs).
- The mean percentage of HFCs was significantly higher in five subjects with a history of liver disease ($P = 0.040$ by Mann-Whitney test), and in 14 workers who reported never having had an x-ray ($P = 0.049$ by Mann-Whitney test).

Table 46. SCEs and HFCs by Group[a]

	Control ($n = 25$)	Monomer ($n = 17$)	Polymer ($n = 31$)
SCEs/cell	6.32 ± 0.87	6.14 ± 0.94	6.47 ± 0.87
HFCs (%)	2.72 ± 4.65	2.71 ± 3.67	3.57 ± 3.67

[a] Values are shown as means ± SD. Subjects lost to analysis: 7 monomer production workers and 3 polymerization workers. We found no significant differences among exposure groups.

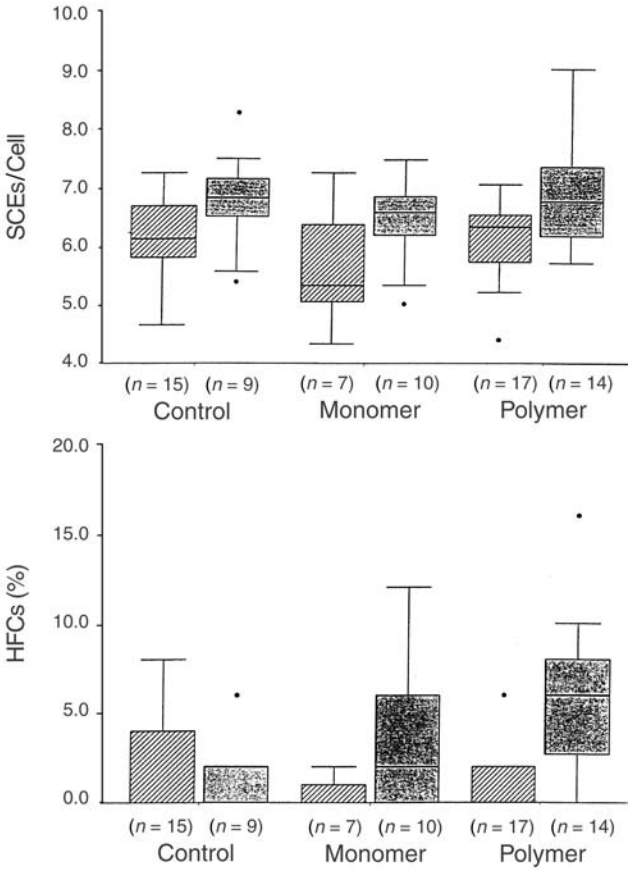

Figure 30. Summary of the data comparing smoking status with SCEs and HFCs. Hatched boxes = subjects who do not currently smoke; gray boxes = subjects who do. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively. Error bars are the highest and lowest values within 1.5 box-lengths from each percentile. The line within each box marks the position of the median value. The dots are values that are more than 1.5 box-lengths from the 75th percentile.

- The mean percentage of HFCs was lower in six workers with a history of kidney disease ($P = 0.042$ by Mann-Whitney test).

In summary, although both of the SCE endpoints were significantly correlated with smoking, no relation to BD exposure was found.

DISCUSSION AND CONCLUSIONS

The cytogenetic endpoints did not prove to be sensitive indicators of BD exposure at the levels encountered in these facilities. Chromosomal aberrations identified by the conventional method, chromosomal changes identified by the newer FISH techniques, and our analyses of SCEs and HFCs produced similarly negative findings. SCEs and some of the aberrations we scored were affected by smoking, which indicates that the methods used could detect exposures known to affect these endpoints.

Chromosomal aberrations determined by either the conventional method or FISH analysis were elevated in the current control group compared with a group of historical control subjects (Rubeš et al 1998; P Rössner, personal communication of unpublished results, 2000). Data for the historical control group were obtained from several sources in the Czech Republic and may not be strictly comparable with industry control subjects. Analysis of questionnaire responses in this study did not reveal a reason for the elevations among control subjects. However, the vigorous exposure assessment in the current study and the availability of surrogate measures of exposure (eg, the urinary metabolites M2 and M1, and the Hb adducts THBVal and HBVal) added certainty that these high values were not due to BD exposure.

Analysis of cytogenetic changes by genotypes showed some associations, but none with the *GST* polymorphisms that have been previously reported (Norppa et al 1995; Uuskula et al 1995). Furthermore, associations with alleles at the *EH113* and *EH139* loci showed conflicting results, with both high-activity and low-activity alleles producing lower frequencies of chromosomal aberrations relative to BD exposures. Much larger studies are needed to make definite statements regarding these genotype associations, but the observations here will be valuable additions to those from future studies and provide direction for investigations in transgenic animals.

Previous results of cytogenetic changes observed in the November 1994 sampling of similar workers in the same petrochemical company were not found in the current study. These differences could not be explained by changes in technology during the years between 1994 and 1998 or by a decrease or increase in the BD exposures; comparing the exposures of monomer production workers between 1994 and 1998 showed no differences in the levels of BD. The only difference between the studies seems to be the sampling period: The 1994 samples were collected in November, the 1998 samples for the current study were collected in May and June. We might speculate that the same level of BD may not affect workers in the same way during the spring season as during the winter. For example, due to a higher temperature in spring, BD could more rapidly evaporate from the workers' breathing zones. The only way to investigate this possibility is to repeat blood sampling during the winter.

CORRELATIONS AMONG BIOMARKERS

We expected that the correlations among the different biomarkers of exposure would be high and that correlations among the biomarkers of exposure and the biomarkers of effect would be low, which is, in fact, what we found. Correlations between the two different urinary metabolites are discussed in the earlier section Biomarkers of Exposure: BD Metabolites in Urine / Results / Correlations Among Urinary Biomarkers and Personal Exposure Monitoring Results; and correlations between the two different Hb adducts are described in the earlier section Biomarkers of Exposure: Hb Adducts / Results / Correlation Between HBVal and THBVal.

CORRELATIONS AMONG BIOMARKERS OF EXPOSURE

We examined the relations among the concentrations of HBVal and THBVal Hb adducts and both urinary metabolites. The Spearman correlation coefficients for each adduct with M2 or M1 are presented in Table 47.

As indicated, both the before-work and the after-work metabolite concentrations were significantly correlated with HBVal Hb adduct concentrations. For the after-work concentrations, the correlations were 0.675 for M1 and 0.682 for M2 ($P < 0.001$ for both). These relations are displayed in Figure 31 with both adduct and metabolite values plotted on logarithmic scales. HBVal Hb adducts also correlated positively and significantly ($P < 0.001$) with net M2 and M1 urinary concentrations (after-work minus before-work), but the correlations were lower than with the after-work concentrations: $r = 0.388$ for net M1 and $r = 0.417$ for net M2. The correlation between the HBVal Hb adduct concentrations and the M2/(M1 + M2) ratios for either before-work or after-work urine samples was positive and statistically significant ($r = 0.360$, $P = 0.001$ for before-work ratio; $r = 0.511$, $P < 0.001$ for after-work ratio).

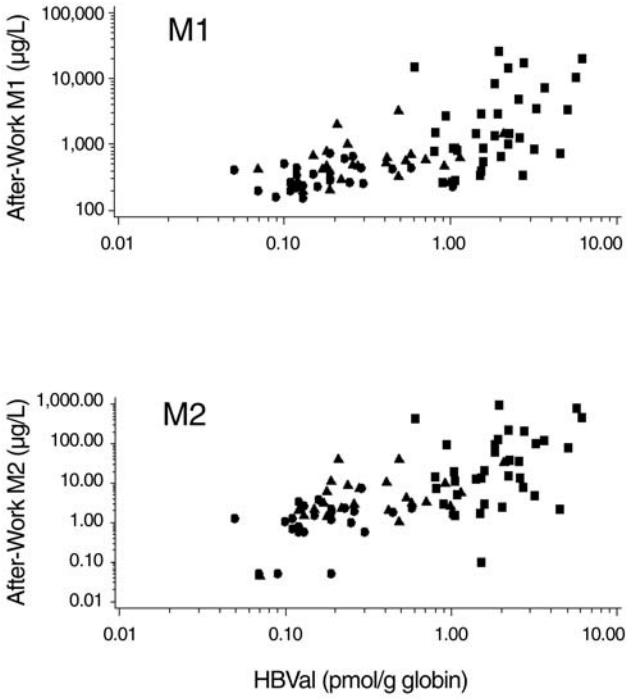

Figure 31. Urinary M2 and M1 metabolites vs HBVal Hb adducts. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

The correlations of THBVal Hb adduct concentrations with before-work and after-work M2 and M1 urine concentrations were positive, and all were statistically significant except the correlation with before-work urinary M1 concentration. Figure 32 shows the adduct and urinary metabolite relations, with both plotted on logarithmic scales. The THBVal Hb adduct concentrations were also significantly ($P < 0.001$) correlated with net urinary M2 and M1 concentrations ($r = 0.476$ and $r = 0.442$, respectively),

Table 47. Spearman Correlations Between Hemoglobin Adducts and Urinary Metabolites

Hemoglobin Adduct	M1 Urinary Metabolite				M2 Urinary Metabolite			
	Before Work ($n = 82$)[a]		After Work ($n = 83$)		Before Work ($n = 82$)[a]		After Work ($n = 83$)	
	r	P	r	P	r	P	r	P
HBVal	0.283	0.010	0.675	< 0.001	0.461	< 0.001	0.682	< 0.001
THBVal	0.192	0.084	0.691	< 0.001	0.445	< 0.001	0.736	< 0.001

[a] One subject's before-work urine sample was not analyzed due to lack of volume.

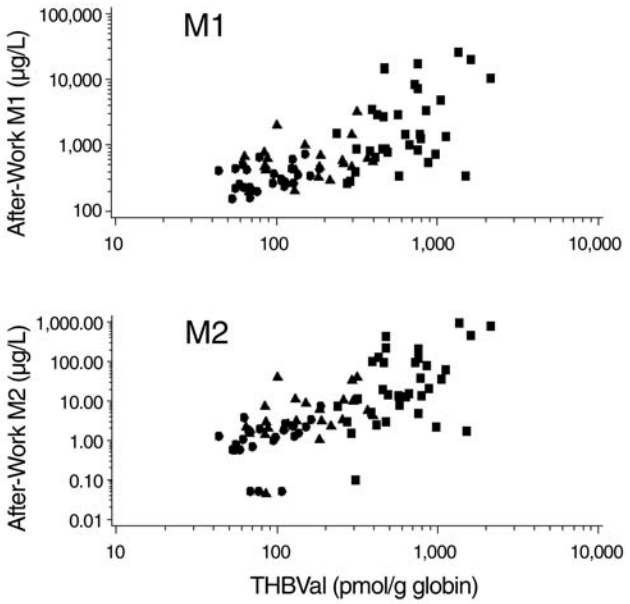

Figure 32. Urinary M2 and M1 metabolites vs THBVal Hb adducts. ● = administrative control subjects; ▲ = monomer production workers; ■ = polymerization workers.

although the correlations are lower than those based on the after-work metabolite concentrations. The Spearman correlations between THBVal Hb adduct concentrations and M2/(M1 + M2) ratios were also positive and statistically significant ($P < 0.001$) for both before-work ($r = 0.421$) and after-work ($r = 0.576$) urine samples.

CORRELATIONS AMONG BIOMARKERS OF EXPOSURE AND BIOMARKERS OF EFFECT

HPRT Mutations Assessed by the Cloning Assay (Mutant Frequencies)

Cloning assay results for 75 of the 83 study subjects (90%) were analyzed. For the remaining eight, the assay was either unsuccessful or yielded an MF of zero. Samples were lost to study for one (4%) administrative control subject, five (21%) monomer production workers, and two (6%) polymerization workers. Correlations of *HPRT* MF values with urinary M2 and M1 metabolites and with HBVal and THBVal adduct concentrations were analyzed and no significant correlations were found (Table 48).

HPRT Mutations Assessed by the Autoradiographic Assay (Variant Frequencies)

Although blood samples were obtained from all 83 study subjects, successful measurement by autoradiographic assay with results suitable for analysis was accomplished on only 49 subjects (59%) for reasons described in the earlier section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Results of the Autoradiographic Assay / Quality of Lymphocyte Samples. An acceptable autoradiographic assay was performed on samples from 18 administrative control subjects, 16 monomer production workers, and 15 polymerization workers.

The Spearman correlations between *HPRT* VF and urinary metabolite concentrations are presented in Table 48 for all 49 study subjects for whom VFs were determined and for the 31 exposed subjects. When all 49 subjects were

Table 48. Spearman Correlations Between *HPRT* Mutant or Variant Frequency and Urinary Metabolites or Hemoglobin Adducts

Measure of BD Exposure	*HPRT* Mutant Frequency[a]				*HPRT* Variant Frequency[b]			
	All Subjects ($n = 75$)		Excluding Controls ($n = 51$)		All Subjects ($n = 49$)		Excluding Controls ($n = 31$)	
	r	P	r	P	r	P	r	P
Urinary Metabolite								
M1 after work	0.033	0.778	0.067	0.962	−0.435	0.002	−0.343	0.059
M2 after work	0.059	0.617	0.058	0.688	−0.394	0.005	−0.346	0.056
Hemoglobin Adduct								
HBVal	0.158	0.175	0.144	0.315	−0.244	0.091	−0.100	0.594
THBVal	0.182	0.118	0.235	0.098	−0.303	0.034	−0.029	0.875

[a] Mutant frequencies were computed only for samples with clonable cells in which MF ≠ 0.

[b] Variant frequencies were computed only for samples containing at least 400,000 evaluatable cells.

included, the correlations with the M2 and M1 metabolite concentrations were negative and significant. When the administrative control subjects were excluded, the coefficients were still negative but were no longer statistically significant.

We also examined correlations between *HPRT* VFs and Hb adduct concentrations (both HBVal and THBVal) (Table 48). All the Spearman correlation coefficients were negative whether the analysis included all relevant study subjects or eliminated the administrative control subjects; the only significant correlation was with THBVal adduct concentrations when all relevant subjects were included in the analysis ($r = -0.303$, $P = 0.034$).

Cytogenetic Changes

Chromosomal Aberrations Identified by the Conventional Method Successful analyses of chromosomal aberrations were accomplished for 82 subjects (99%). Only one sample, from a monomer production worker, was lost to analysis. The endpoints measured were the percentage of aberrant cells and the number of breaks/cell. Analyses of the relations among both of these measures of chromosomal aberration and urinary M2 and M1 concentrations and HBVal and THBVal Hb adduct concentrations revealed no significant correlations regardless of whether the administrative control subjects were included in the analysis (Table 49).

Chromosomal Changes Identified by FISH Successful analysis by FISH was accomplished for 78 of the subjects (94%). Samples lost to analysis included one (4%) from an administrative control subject, one (4%) from a monomer production worker, and three (9%) from polymerization workers. None of the five cytogenetic endpoints determined by FISH were significantly correlated with urinary M2 or M1 metabolite concentrations or with THBVal Hb adduct concentrations (Table 49). HBVal Hb adduct concentrations were significantly correlated with reciprocal translocations ($r = 0.260$, $P = 0.021$) but not with any of the other endpoints.

SCEs Assays for SCEs were successfully completed for 73 subjects (88%). Assays were unsuccessful for seven (29%) monomer production and three (9%) polymerization workers. Spearman correlation coefficients were computed to assess relations between SCEs or percentage of HFCs and urinary M2 or M1 metabolite concentrations or HBVal or THBVal Hb adduct concentrations. We found no significant correlations with any of these surrogate measures of BD exposure, regardless of whether the administrative control subjects were included in the analysis (Table 49).

Table 49. Spearman Correlations Between Cytogenetic Endpoints and Urinary Metabolites or Hemoglobin Adducts[a]

		Urinary Metabolite				Hemoglobin Adduct			
		M1 After Work		M2 After Work		HBVal		THBVal	
Cytogenetic Endpoint[b]	*n*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Aberrant cells (%)	82	0.147	0.187	0.140	0.211	0.083	0.461	0.038	0.736
Breaks/cell	82	0.156	0.161	0.143	0.199	0.068	0.542	0.012	0.917
Chromosomal aberrations (FISH) (‰)	78	−0.027	0.816	−0.133	0.246	0.136	0.235	0.045	0.698
Translocations (FISH) (‰)	78	0.019	0.867	−0.109	0.343	0.150	0.190	0.042	0.714
Reciprocal translocations (FISH) (‰)	78	0.156	0.173	0.032	0.784	0.260	0.021	0.125	0.277
Conjunctions (FISH) (‰)	78	−0.015	0.893	−0.145	0.206	0.141	0.219	0.037	0.746
F_G/100 (FISH)	78	0.021	0.858	−0.108	0.349	0.152	0.184	0.044	0.704
Sister chromatid exchanges/cell	73	0.158	0.182	0.052	0.662	0.082	0.488	0.046	0.701
High frequency cells (%)	73	0.186	0.114	0.122	0.305	0.228	0.053	0.202	0.086

[a] Correlations were based on all subjects with available data (see *n* column). Very similar results were obtained when control subjects were excluded.

[b] Chromosomal changes analyzed via FISH and noted as (‰) are presented per 1000 metaphases.

Table 50. Spearman Correlations Between Cytogenetic Endpoints and *HPRT* Mutations[a]

	Mutant Frequency via Cloning Assay			Variant Frequency via Autoradiographic Assay		
Cytogenetic Endpoint[b]	*n*	*r*	*P*	*n*	*r*	*P*
Aberrant cells (%)	74	0.045	0.703	49	0.015	0.921
Breaks/cell	74	0.066	0.575	49	0.035	0.812
Chromosomal aberrations (FISH) (‰)	71	−0.064	0.596	47	0.251	0.088
Translocations (FISH) (‰)	71	−0.071	0.557	47	0.201	0.175
Reciprocal translocations (FISH) (‰)	71	0.041	0.732	47	0.077	0.608
Conjunctions (FISH) (‰)	71	−0.077	0.524	47	0.179	0.228
F_G/100 (FISH)	71	−0.068	0.575	47	0.200	0.177
Sister chromatid exchanges/cell	66	−0.004	0.977	42	−0.065	0.685
High frequency cells (%)	66	0.031	0.805	42	−0.286	0.067

[a] Correlations were based on all subjects with available data (see *n* columns). Very similar results were obtained when control subjects were excluded.

[b] Chromosomal changes analyzed via FISH and noted as (‰) are presented per 1000 metaphases.

Correlations Among the Biomarkers of Effect: *HPRT* Mutations and Cytogenetic Endpoints

HPRT mutations were determined by both the autoradiographic and cloning assays. Mutations were not associated with BD exposure regardless of the method used to assess them. Several measures of cytogenetic changes were also analyzed. As noted earlier, none of the cytogenetic endpoints was significantly associated with BD exposure levels, whether assessed by direct measurements or surrogate markers of exposure.

Table 50 shows that the *HPRT* mutations, expressed as VF or MF, were not significantly correlated with any of the cytogenetic endpoints measured. Thus, not only were these biomarkers of genetic effects unrelated to BD exposures, they were also unrelated to each other.

CONCLUSIONS

All of the biomarkers of exposure that we evaluated proved to be valid surrogate measures of BD exposure. It is not surprising, therefore, that these biomarkers (M2 and M1 urinary metabolites and the HBVal and THBVal Hb adducts) were all highly correlated with each other. To the contrary, none of the biomarkers of effect that we evaluated proved to be valid surrogate measures of BD exposure. Thus, the lack of positive correlation between any of the biomarkers of effect and any of the biomarkers of exposure is consistent with and supports this observation. This is further reflected in the lack of correlation between either of the two measures of *HPRT* mutations and any of the several cytogenetic endpoints evaluated.

DISCUSSION AND CONCLUSIONS

This was a transitional epidemiologic study in which we evaluated a continuum of biomarkers for their sensitivity to detect and quantify BD exposures in the occupational setting.

EXPOSURE ASSESSMENT

The foundation of the study was an extensive exposure assessment phase that was critical for the study to succeed. The scheme was designed to measure each worker's actual BD exposure as accurately as possible and to allow for day-to-day variations. Over a 60-day interval, each worker's personal BD exposure was measured eight to ten times, and his contact with styrene, benzene, and toluene was determined. Workplace area concentrations of all of these agents were also measured.

An important consideration in designing our exposure assessment scheme was the uncertain patterns of exposure experienced by the workers. Exposure to BD might be stable and constant over time or it may be episodic or variable. The latter possibility raised the problem of appropriately timing the exposure measurements to correspond to the biomarkers being evaluated because each biomarker has a characteristic time of expression and duration of persistence. Because several biomarkers were being evaluated, several expression times and persistence intervals needed to be considered. These kinetic factors were built into the design of our exposure assessment scheme.

The biomarkers evaluated in the current study are shown in Table 51 with their levels of specificity and probable expression and persistence times. The metabolites measured in urine are listed as specific for BD exposure. However, the relatively high background levels of M1 in the administrative control subjects in this study raises questions about this specificity. It is possible that some of the M1 originates from an endogenous source. Similarly, because we assumed that urinary metabolites are excreted within hours of exposure, we listed a short persistence duration in Table 51. As discussed below, however, our findings also cast doubt on this assumption.

The biomarkers measured in blood have longer expression times. First, hemoglobin adducts reflect cumulative exposures over the past four months. They disappear at a rate of approximately 1% per day after exposure ceases. As noted, although the THBVal hemoglobin adducts reflect in vivo concentrations of the BDO-diol metabolite, they too were frequently detectable at appreciable concentrations in individuals not exposed to BD. Therefore, they may have some exogenous (or even endogenous) source other than BD. For this reason, hemoglobin adducts are listed as "somewhat specific" in Table 51. Second, newly induced *HPRT* mutations in lymphocytes evolve for weeks to months before they reach their full expression and the optimal time to recover them from blood; they may persist for months to years. Third, several of the chromosomal changes measured in this study result from underlying DNA damage that occurred in vivo (perhaps within hours to days of a genotoxic exposure) but that can only be assessed as irreversible chromosomal changes by removing the cells from the body and cultivating them in vitro. Exceptions are the stable translocations, which most likely arise in vivo and, like the mutations, take weeks to months to reach their maximal appearance. Although they begin to decline soon after the exposure, most chromosomal changes persist for days to months and stable translocations persist for years.

Table 51. Biomarkers Assessed in BD Study

Source	Biomarker	Time of Expression	Duration of Persistence	Specificity
Urine	M2 urinary metabolite	Hours	Hours	Very
Urine	M1 urinary metabolite	Hours	Hours	Somewhat
Blood	Hemoglobin adducts	Cumulative	Months	Somewhat
Blood	*HPRT* mutations via cloning	Months	Months to years	Nonspecific
Blood	*HPRT* mutations via autoradiography	Weeks	Months to years	Nonspecific
Blood	Chromosomal aberrations via the conventional method	Hours	Days to months	Nonspecific
Blood	Chromosomal changes via FISH[a]	Hours	Months to years	Nonspecific
Blood	Sister chromatid exchanges	Hours	Days to weeks	Nonspecific

[a] Translocations persist longer than other aberrations.

Because of these timing variables, exposure measurements were obtained at times relevant to each of the biomarkers being evaluated. Exposure measurements at an intermediate time (weeks to months) before blood sample collections were optimal for evaluating hemoglobin adducts, *HPRT* mutations, and stable chromosomal translocations; exposure measurements at a shorter time interval (days to weeks) before sample collection were optimal for evaluating most of the other chromosomal changes; and exposure measurements immediately (hours) before urine sample collection were optimal for evaluating the short-lived urinary metabolites. Thus, we spaced exposure measurements semirandomly throughout the 60-day exposure assessment period and ensured that at least one of the measurements was taken early in the period.

The average BD exposures of the workers involved in this study were quite moderate and indicate well-run facilities. The group means were within the range of those cited in other studies (Tates et al 1996; Ward et al 1994, 1996b; Šrám et al 1998; Hayes et al 2000; Ma et al 2000) and monomer production workers had, on average, lower exposures than polymerization workers. Of note is the wide day-to-day variability of individual worker exposure levels in both exposed groups. It is possible that, even though multiple measurements of exposure were made during the 60-day period (covering about 12–15% of each worker's work time), some "high-exposure" days were missed, possibly underestimating some workers' average exposures. As expected, the administrative control subjects had very low-level (near outdoor ambient) BD exposures. Although only single measurements were made for each administrative control subject, the narrow range of interindividual levels suggests that intraindividual variability also was small.

The exposure concentrations of styrene, benzene, and toluene were quite low, although styrene levels were significantly higher in the polymerization workers. (Although we considered measuring dimethyldithiocarbamate in this study, we didn't because it had never been used at these facilities.) Workplace area measurements of all agents were consistent with the workers' exposures as determined from personal monitoring. The plan to measure peak levels of BD exposure by mini-photoionization chambers could not be accomplished primarily because of equipment failure. The loss of this important metric must be considered a deficiency. Despite this, however, the current exposure assessment is the most extensive ever achieved in the BD industry and the only one in a transitional epidemiologic study to take into account the kinetics of individual biomarkers.

STUDY SUBJECTS

Questionnaire and interview responses indicated that the exposed and control groups in this study were well balanced with regard to age and smoking habits but not education; furthermore, the assignment of interviewers to exposure groups may have introduced bias to the information obtained. Questionnaires revealed some differences among exposure groups in terms of diet, health, and medication variables. We found no significant differences among the exposure groups studied for any of the metabolic genotypes determined (ie, *CYP2E1*, *GSTM1* and *GSTT1*, and the EH and ADH isoforms).

URINARY METABOLITES

The urinary M2 and M1 metabolite concentrations determined in this study correlated well with BD exposures, both at the group level and for individual workers. The metabolites were assayed from aliquots of urine samples obtained before and after workshifts on the first of three days at the end of the exposure assessment period. Their concentrations were not influenced by smoking. These metabolites proved to be sensitive biomarkers for detecting BD exposure in humans.

In addition to their value as biomarkers of exposure, the urinary metabolite concentrations determined in this study proved instructive regarding BD metabolism in humans. The differences between the monomer production and polymerization workers were 6.1-fold in this study for metabolite concentrations of M2 and 12.7-fold for concentrations of M1, whereas the difference in BD exposure levels was only 2.7-fold (Table 52). This is consistent with threshold effects whereby increasing concentrations of BD stimulate various metabolic pathways differently, at least for detoxification. However, there may be simpler explanations for these observations. Both the metabolite and the BD exposure data are highly skewed and the proportionality of the means depends on how they are distributed. Comparing the medians shows the relative increases to be more similar. Also, the urinary metabolites reflect recent exposures, whereas BD measurements are averages over a 60-day period. High BD exposures on the day of urine collection could result in high metabolite values and, hence, a high mean, whereas the mean BD exposure level is less influenced by single high-exposure measurements.

It has been assumed that both the M2 and M1 metabolites are short lived, persisting for a matter of hours. The plan in this study therefore was to measure BD exposures by personal monitors daily on the last three days of the exposure assessment period and to obtain urine samples

Table 52. Comparisons of BD Exposure and Biomarkers Among Groups[a]

	Control (n = 25)	Monomer (n = 24)	Polymer (n = 34)	Polymer Greater Than Monomer by a Factor of [b]
BD Exposure[c]	0.026 ± 0.030	0.643 ± 2.056	1.760 ± 4.692	2.7
M1 after work[d]	353 ± 157	764 ± 728	4647 ± 6630	6.1
M2 after work[d]	1.70 ± 1.54	9.44 ± 12.97	120.17 ± 228.17	12.7
HBVal[e]	0.224 ± 0.205	0.466 ± 0.452	2.230 ± 1.399	4.8
THBVal[e,f]	94.77 ± 38.71	178.73 ± 101.31	716.70 ± 425.72	4.0

[a] Values are presented as means ± SD.

[b] Comparison values were drawn from the Discussion and Conclusions section.

[c] From Table 10. Exposure is reported in mg/m^3.

[d] From Table 19. Urinary metabolites are reported in μg/L.

[e] From Table 23. Adducts are reported as pmol adduct/g globin.

[f] The mean THBVal concentrations were 321-fold to 423-fold higher than the mean HBVal concentrations.

before and after workshifts on each day. However, because of workers' schedules and the need for a two-day work-free washout period before urine collection, this plan was not feasible for the majority of workers. Samples (and therefore urinary metabolite data) were obtained, therefore, for some workers with short time intervals between BD exposure measurements and urine collection, and from other subjects with longer time intervals between the two.

Our data collection plan and analysis provided some findings that challenge the assumptions generally held. First, urinary metabolite concentrations correlated as well with the 60-day average BD exposure levels as they did with the same-day exposure levels. Second, and more surprising, the net urinary metabolite concentrations (after-work minus before-work concentrations) were negative for many of the exposed workers. Third, we found positive correlations between *before*-shift metabolite concentrations and BD exposures, especially for the polymerization workers, who had the highest exposure levels. It is noteworthy that these positive correlations were obtained for both direct (personal monitoring and workplace area concentrations) and surrogate (eg, hemoglobin adducts as biomarkers) measurements of BD exposure. Negative net metabolite concentrations had been expected for administrative control subjects for whom work-related BD exposures were near outdoor ambient levels. But this was not expected for the monomer production or polymerization workers, who clearly had occupational exposures. In these workers, a negative net metabolite concentration must mean that the persistence of the M2 and M1 metabolites in humans is longer than assumed; this conclusion is also supported by the finding that two work-free days was not an effective washout period, demonstrated by the positive correlations between before-shift urinary metabolite concentrations and BD exposure.

Other features of BD metabolism in humans are revealed by these urinary metabolite data. The urinary M1 concentrations were much higher than the M2 concentrations, with M2 constituting only about 1% of the total metabolites. This is consistent with hydrolysis being the major pathway for BD detoxification in humans (as in the rat), rather than the conjugation detoxification pathway, which predominates in the mouse (Henderson et al 1996). The ratio M2/(M1 + M2) (which was approximately 0.01 in this study) may be taken as a measure of the minor conjugation detoxification pathway in humans. Although the differences among the exposure groups did not reach statistical significance, this ratio was lowest in the control group, higher in the monomer group, and highest in the polymer group, the same order as BD exposure levels. This suggests that either the hydrolytic pathway becomes saturated or the conjugation pathway becomes stimulated. This increasing conjugation with increasing BD exposure is blunted, however, in individuals who have either the *GSTM1*-null or *GSTT1*-null genotype; the magnitude of difference between positive and null genotypes was statistically significant for *GSTM1* (see Table 21). This would seem to favor stimulation of conjugation with GSH as the explanation for the increasing M2/(M1 + M2) ratios, which is not possible to the same degree in individuals who have a null genotype at one of these loci.

It has also been postulated that the formation of M1 involves ADH (Sabourin et al 1992; Kemper et al 1996); but in this study, neither the excretions of M2 and M1 nor the

ratio M2/(M1 + M2) showed any correlation with *ADH* genotypes. However, these findings do not exclude a possible role for ADH in the formation of M1. For *ADH2*, which was one of the *ADH* polymorphisms we studied, very few workers carried the variant allele.

Another unexpected observation in this study was an association between subjects with the *CYP2E1 D/D* genotype and increased production of the M2 metabolite, as demonstrated by higher M2/(M1 + M2) ratios relative to BD exposure levels. The significance of this remains to be defined; comparative studies of transgenic rodents may help to clarify the finding and further elucidate the metabolic pathways of BD.

The BD urinary metabolite results illustrate how molecular epidemiologic findings in humans can be used, along with animal data, for the overall toxicologic evaluation of a chemical. From the urinary metabolite data, it might be expected that even if BDO_2 is formed in humans, it is rapidly hydrolyzed to BDO-diol, or erythritol, or both, or it may be further metabolized by conjugation with GSH (Boogaard and Bond 1996; Boogaard et al 1996). Therefore, very little BDO_2 should be available to react with DNA. Unfortunately, we have no data on the actual excretion of the BDO_2 metabolite. However, the data on hemoglobin adduct formation in this study is consistent with the interpretation derived from the urinary M2 and M1 metabolite results.

HEMOGLOBIN ADDUCTS

We evaluated two hemoglobin adducts: The HBVal hemoglobin adduct provides a measure of in vivo concentrations of BDO (the first oxidation product of BD) and the THBVal hemoglobin adduct measures in vivo concentrations of BDO_2 and BDO-diol (however, current evidence about THB-DNA adducts indicates that BDO-diol is the predominant contributor to the THBVal hemoglobin adduct [Pérez et al 1997; Koivisto et al 1999]).

The group mean concentrations of both hemoglobin adducts were significantly higher in the monomer group than in the control group, and significantly higher in the polymer group than in either of the other two exposure groups. This is the order of increasing BD exposures, which indicates that the adduct concentrations clearly reflect exposure levels. As we found with the urinary M2 and M1 metabolite concentrations, the increases in mean hemoglobin adduct concentrations in the exposure groups departed somewhat from linearity: the 2.7-fold difference in BD exposure between the monomer and polymer groups resulted in a 4.8-fold difference in HBVal concentrations and a 4.0-fold difference in THBVal concentrations (Table 52). This nonlinear relation is characterized by the exponential equation relating workers' adduct concentrations to their measured BD exposures, and, as we found with urinary metabolites, is consistent with a threshold effect in metabolism. These increases in adduct concentrations more closely reflect the increases in 60-day average BD exposure levels than do the increases in urinary metabolite concentrations. Because hemoglobin adducts reflect cumulative BD exposures, we expected this on statistical grounds.

The mean THBVal hemoglobin adduct concentrations were from 368-fold to 600-fold higher than the mean HBVal hemoglobin adduct concentrations; this relation presumably reflects the predominance of the hydrolytic pathway in humans resulting in a much greater in vivo accumulation of BDO-diol than BDO. The difference between THBVal and HBVal concentrations has also been observed for DNA adducts in rodents at low, but not high, BD exposure levels; those observations have been interpreted as the metabolism of BDO to BDO_2 or BD-diol reaching saturation (Koc et al 1999). It is noteworthy that the humans in this study were exposed to lower BD levels than the rodents studied, but the ratios between the hemoglobin adducts were higher in the humans than in the rodents. This is consistent with a report that, at high concentrations, BD inhibits further oxidation of BDO (Dahl and Henderson 2000). Despite the differences in absolute concentrations, the HBVal and THBVal hemoglobin adducts were highly correlated with each other in this study.

Both the HBVal and the THBVal adduct concentrations also were highly correlated with BD exposures for individual workers' average exposures (Spearman coefficients of 0.676 for HBVal and 0.737 for THBVal). These coefficients are remarkable given that BD exposures were measured over only 12% to 15% of the time for which the adducts showed cumulative internal doses. It well may be that the correlation coefficients would be even greater if BD exposure had been measured over the total time and that the adducts are, in fact, better indicators of exposure than are the external BD measurements. Nevertheless, in equations that were developed to relate the adducts to BD exposure levels, 72% to 77% of the variability in workers' adduct concentrations could be explained by differences in their measured BD exposures. These equations should be tested in subsequent studies that include even more complete exposure measurements with the goal of developing a biological exposure index using hemoglobin adducts.

The current study showed quite high THBVal adduct concentrations in the administrative control subjects who had virtually no exogenous BD exposures (see Table 23). The concentrations of this adduct in administrative control subjects were also higher in this study than previously

reported for control subjects in China (Hayes et al 2000). The reasons for this are currently unknown; it is possible that the THBVal adducts are nonspecific and thus reflect other exogenous exposures or reactions with products of endogenous metabolism.

Genotypes for *CYP2E1*, *GST*, *EH*, and *ADH* polymorphisms were obtained for all workers. Although the *CYP2E1*, *GST*, and *ADH* polymorphisms did not show associations with hemoglobin adducts, one of the *EH* polymorphisms did. Workers with the *Arg139*/*Arg139* homozygous genotype (the allele with increased activity) had significantly lower HBVal and THBVal adduct concentrations relative to BD exposure levels than did workers with the other two genotypes of this polymorphism. Also, workers with the *His139*/*Arg139* heterozygous genotype at this locus actually had higher HBVal and THBVal concentrations than either of the other two genotypes. The decreased concentration of HBVal adducts derived from BDO might be explained by an increase in hydrolysis of BDO in workers with the *Arg139*/*Arg139* genotype. However, this does not explain the greater HBVal adduct production in workers with the *His139*/*Arg139* genotype compared with workers with the *His139*/*His139* genotype.

Furthermore, other observations are not in accord with this rationale; for example, the increased concentrations of the THBVal adducts in workers heterozygous for *His139*/*Arg139* or homozygous for *His139*/*His139* would not support this line of reasoning because THBVal adducts are thought to derive primarily from BDO-diol, production of which is dependent on the hydrolytic pathway (see Figure 1). The increased production of THBVal adducts in workers with the *His139*/*His139* or the *His139*/*Arg139* genotype might reflect the small proportion of the THBVal adducts derived from BDO_2. However, this does not explain why workers heterozygous for *His139*/*Arg139* produced significantly more THBVal adducts than workers homozygous for *His139*/*His139*. Although the numbers and distributions of the workers with the different *EH139* genotypes are far too small to permit definitive assessments of these possibilities (especially that only four workers were homozygous for *Arg139*/*Arg139*), the current study does provide genotypic data that may be combined with findings in other molecular epidemiologic studies and may suggest further animal studies in rodents genetically modified in these metabolic pathways.

The present study has demonstrated that both hemoglobin biomarkers can be used to monitor groups of workers exposed to low levels of BD. The excellent correlation between industrial hygiene measurements and these hemoglobin adducts permits researchers to rank individuals by exposure and then correlate that ranking with other nonspecific biomarkers of genetic effects such as somatic mutations and cytogenetic changes. This correlation allows researchers to examine exposure-response relations within an exposed population and eliminate confounding from problems arising in control groups. Such ranking of exposed individuals was used in the comparative genotoxicity studies of the present investigation.

GENOTOXICITY

The remaining biomarkers evaluated for their sensitivity as indicators of BD exposure were all measures of genetic changes. Conventionally, these have been considered to be biomarkers of effect (although cytogenetic markers have been the gold standards for measuring acute recent exposures to ionizing radiation). None of these measures showed positive responses at the exposure levels encountered in this study. Because biomarkers that reflect irreversible genetic damage are more removed from the external exposure than are urinary metabolites or hemoglobin adducts, these results are not unexpected despite the occasional reports of positive genotoxic responses in BD-exposed workers (see Table 1).

Neither the cloning nor the autoradiographic assay for *HPRT* mutations in T lymphocytes showed a BD effect. The autoradiographic assay had produced positive findings in previous studies, and the laboratory that performed the assay in the current study had also conducted the earlier studies (Ward et al 1994, 1996b). Conditions of the studies differed however. First, in the current study, blood samples were obtained and the lymphocytes were fractionated and cryopreserved in a facility different from the one that performed the assay. Second, several days elapsed before the dry shippers sent from Prague reached the laboratory in Galveston for testing. A successful assay was possible for only 49 (60%) of the 82 samples and a disproportionate loss of usable samples occurred in the most heavily exposed (polymerization) worker group (only 45% of the samples provided usable information). Third, inspection of the data revealed that the *HPRT* variant frequency values for the control group were 8-fold to 10-fold higher than this laboratory had ever recorded for unexposed healthy American adults. Because biomarkers of genetic effects are nonspecific, this elevation in the control group mean could be due to a variety of factors, although none was obvious from analyzing the questionnaire data. Given the exposure measurements, the urinary metabolite results, and the hemoglobin adduct concentrations, however, we are certain that BD exposure was not responsible. Furthermore, the Spearman correlation coefficients relating variant frequency values to BD exposure concentrations remained negative even when the control group was eliminated from

the analysis. Results of the autoradiographic assay and the correlation coefficients remained negative even if variant frequency values were compared with BD exposure measurements made in the interval 70 to 50 days before blood collection, a duration of time that should have been optimal for the expression of *HPRT* mutations. Technical misreading of autoradiographic slides could not have contributed to these negative results because the researchers reading the slides were blinded as to the subject and exposure information in two laboratories with good concordance between them. In addition, *HPRT* variant frequencies were unrelated to worker genotypes. Thus the reason for negative results in the current study but positive results in earlier studies remains unexplained.

HPRT mutations as measured by the cloning assay were also unrelated to BD exposures. Here, however, the mutant frequency values for the administrative control subjects, which did not differ from those of exposed workers, were in accord with expectations. These negative findings are consistent with past studies that failed to find a relation between *HPRT* mutations and BD exposures (Tates et al 1996); one of those studies was conducted by the laboratory that performed the current assays. Certain findings in the current study, such as a positive age effect and a positive correlation between *HPRT* mutant frequencies and cumulative smoking history, give confidence that the assay proceeded satisfactorily. BD exposures did appear to reduce the nonselection cloning efficiencies in the cloning assays, which could be a manifestation of cytotoxicity. This is unlikely, however, because an earlier study in China (Hayes et al 2000) reported an actual increase in lymphocyte blood counts associated with BD exposure.

When *HPRT* mutations have been compared with other endpoints as dosimeters for detecting ethylene oxide exposure, they were found to be the least sensitive (Tates et al 1991a). Cytogenetic changes, such as sister chromatid exchanges and chromosomal aberrations, were more sensitive dosimeters than *HPRT* mutations, even though chromosomal aberrations are also a measure of genetic effects. This is probably because the *HPRT* mutation assays can assess changes in only a small portion of the genome, whereas cytogenetic alterations can be detected throughout, providing a much larger target for recognition.

We also analyzed molecular mutational spectra to ensure that no BD-induced mutational changes had been missed due to variability in the quantitative assay. We detected no significant differences in mutations between exposed and unexposed subjects; the mutational spectrum for the unexposed administrative control subjects was not statistically different from the spectra for the two BD exposure groups. Furthermore, the molecular spectrum observed across all subjects in the present study did not differ significantly from spectra derived from historical datasets for humans not exposed to mutagens. Although we did observe some novel mutations (see the earlier section Biomarkers of Effect: *HPRT* Mutational Spectra) thus adding to these datasets, our analyses of the *HPRT* mutational spectra provided no support for the hypothesis that BD induces genetic mutations.

Finally, *HPRT* mutant frequencies were unrelated to the metabolic genotypes we had identified.

Considerations of sample size suggest that the number of workers evaluated in the current study would have a 90% probability of detecting a 70% increase in *HPRT* mutations in the exposed workers compared with unexposed control workers (Robinson et al 1994). Therefore, the power of this study to detect an effect on *HPRT* mutations was acceptable.

Despite the knowledge that chromosomal changes have greater sensitivity than genetic mutations for detecting other chemical exposures, none of the cytogenetic endpoints evaluated in this study showed a BD effect. Sister chromatid exchanges are usually sensitive indicators of chemical exposures, but we found no BD-related responses in this study. Chromosomal aberrations, whether assessed by the conventional method or by FISH techniques, were similarly negative. Sister chromatid exchanges were affected by smoking, as were some of the aberrations that were scored, which indicates that the methods were capable of detecting exposures known to affect these endpoints.

Unlike *HPRT* mutations, metabolic genotypes did appear to influence cytogenetic responses. Both the percentage of aberrant cells and the number of breaks/cell determined by the conventional method showed associations with metabolic genotypes. The most consistent relations were with the *ADH2* polymorphisms, which were associated with differences in both endpoints. Subjects with the *Arg47*/*Arg47* genotype at this locus had significantly lower mean values for the percentage of aberrant cells and breaks/cell than did the subjects with the *Arg47*/*His47* genotype (see Table 42; no subjects homozygous for *His47*/*His47* were identified). Regression analyses, however, provided no evidence that these genotypic differences were related to BD exposures.

Although we found no statistically significant genotypic differences in percentage of aberrant cells or breaks/cell among the *EH* polymorphisms and no significant genotype–by–exposure-group interactions (see Table 42), regression analyses of the cytogenetic endpoints on ln[BD-exposure-level–by–genotype] did reveal some statistically significant associations. At the *EH113* locus, workers

homozygous for *Tyr113*/*Tyr113* had lower slopes than the workers with either the *His113*/*His113* or the *Tyr113*/*His113* genotype for percentage of aberrant cells and breaks/cell; but those slope differences were statistically significant only between the workers with *Tyr113*/*Tyr113* and those with *Tyr113*/*His113* genotypes (see Figure 27). *Tyr113* is the allele with higher activity at this locus and would be expected to be associated with less genotoxicity due to greater detoxification. However, the lowest EH enzyme activity should be in the workers homozygous for *His113*/*His113*. Although these individuals did show more genetic effects than workers homozygous for *Tyr113*/*Tyr113*, the differences were not significant.

At the *EH139* locus, however, workers with the *Arg139*/*Arg139* genotype had a significantly greater slope for percentage of aberrant cells than did the workers with either the *His139*/*His139* or the *Arg139*/*His139* genotype (see Figure 26). The lower production of aberrant cells was associated with either homozygosity or heterozygosity for the lower-activity allele at the *EH139* locus, which, in this case, is the variant *His139* allele. Overall, these results comparing the *EH* genotypes are conflicting. The *EH* genotypes at the *113* locus associated with the higher enzyme activity showed lower genotoxicity as expected; but the reverse was seen for the *139* locus.

No significant associations were observed between either the percentage of aberrant cells or breaks/cell and *CYP2E1*, *ADH3*, *GSTM1*, or *GSTT1* genotypes in this study. The lack of association with the *GST* polymorphisms is in contrast to other studies described in the earlier Overview section.

All exposure group mean chromosomal changes determined by FISH (ie, chromosomal aberrations, translocations, reciprocal translocations, conjunctions, and F_G/100) were significantly lower in the two subjects with the *CYP2E1 C1*/*C1* genotype than in the workers with the *C1*/*C2* genotype (see Table 44). In addition, we found a significant exposure-group–by–genotype interaction between the *EH113* polymorphisms for chromosomal aberrations ($P = 0.036$), translocations ($P = 0.034$), conjunctions ($P = 0.047$), and F_G/100 ($P = 0.034$). The administrative control subjects and the polymerization workers with the *Tyr113*/*His113* heterozygous genotype showed *higher* frequencies of these chromosomal changes than the workers with either of the homozygous genotypes in their respective exposure groups, whereas monomer production workers with the *Tyr113*/*His113* heterozygous genotype showed *lower* frequencies than workers with the homozygous genotypes in their exposure group (see Table 45). The biological significance, if any, of these associations is unknown. We identified no other associations among other genotypes and chromosomal changes determined by FISH analyses.

Finally, neither sister chromatid exchanges nor high-frequency cells showed a relation with metabolic genotypes.

Similar to *HPRT* mutations detected by autoradiography, at least one earlier study of BD-exposed workers had shown an increased frequency of chromosomal aberrations as determined by the conventional method (Šrám et al 1998). Another reported an increased frequency of these events, but only in exposed workers of the *GSTT1*-null genotype (Sorsa et al 1996). Both earlier studies included workers from the same facilities that we studied. Another similarity between the current cytogenetic and autoradiographic *HPRT* results is that values for the control group were higher than expected for both. Chromosomal aberrations, determined by either the conventional method or FISH analysis, were elevated in the current unexposed control group compared with historical control data. However, the magnitude of this difference was not great, and the historical control data were obtained from several sources and may not be strictly comparable to industry control subjects. As for the *HPRT* mutations, analysis of questionnaire responses in this study did not reveal a reason for the elevations among administrative control subjects; again, however, we can be certain that BD exposure was not the cause.

Although the cytogenetic findings in this study must be considered negative for a BD effect, we cannot exclude the possibility that the associations with *EH* genotypes may indicate a sensitive human population. This is an important caveat because of the strong relation between BDO_2, chromosomal changes, and carcinogenicity in the rodent systems. Further studies are required to investigate this observation that may be relevant for making human cancer risk assessments.

CONCLUSION

With 83 workers, this study had at least an 80% chance of detecting correlations of 0.30 or greater among biomarkers and levels of exposure or between two biomarkers. This was sufficient statistical power for the objectives of the study because biomarkers with lower correlations would not be sensitive indicators of exposure. As noted, the study had adequate power to detect an increase in *HPRT* mutations (70% or higher) or in cytogenetic changes, but not to test for smaller increases in these biomarkers. However, the clear lack of response in either biomarker argues that, at these exposure levels, BD does not produce

detectable genetic effects in the worker population as a whole. This observation must be reconciled with the few earlier studies that did indicate genotoxicity, but it is in accord with several previous negative studies. The exposure assessment in the current study was the most extensive ever accomplished in the BD industry, and some of the discordance in genotoxicity results may relate to this. We must emphasize that the laboratories that reported positive genotoxicity in earlier studies are the ones that performed the current assays, although on different populations for *HPRT*.

Even though the conflict between positive and negative results remains, the current study adds to the weight of evidence against genotoxicity for the population as a whole at the low exposure levels encountered in the modern BD industry. It does not offer new evidence for upgrading the classification of BD to a known human carcinogen (Class 1; IARC 1999) nor does it support the EPA's recent classification of BD as a known human carcinogen by inhalation (EPA 2002). This study has amply demonstrated the power of using biomarkers of exposure together with biomarkers of genetic effects to interpret the biomarkers of genetic effects. Studies of workers exposed to higher levels of BD would be needed to clarify the question of genetic change, which is the important qualitative nontumor endpoint for making human cancer risk assessments for a putative carcinogenic agent with a known genotoxic mode of action in animals.

This investigation, therefore, was a successful transitional epidemiologic study that evaluated a continuum of biomarkers for their sensitivity in detecting and measuring BD exposures in occupationally exposed humans. The urinary M2 and M1 metabolites proved to be sensitive indicators of exposure, but the HBVal and THBVal hemoglobin adducts were even more so. The adducts performed so well in this regard that serious consideration should be given to using them as surrogate measures of exposure and an effort should be made to develop them for specifying a biological exposure index for humans. Neither the urinary metabolite nor the hemoglobin adduct concentrations were affected by smoking. Metabolic genotypes, however, did influence BD metabolism as indicated. The biomarkers that detect genetic effects such as somatic mutations and chromosomal changes did not prove to be sensitive indicators of BD exposure in the overall population at the levels encountered in these facilities. Data such as these can now be considered in the context of the toxicology of BD, specifically for evaluating the physiologically based pharmacokinetic models developed in animals as to their relevance for humans.

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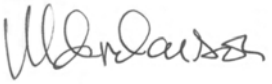
APPENDIX A. HEI Quality Assurance Report

The conduct of this study was subject to periodic, independent audits by Arthur D Little, Inc. The auditor's background includes toxicology, analytical chemistry, environmental health and safety, and occupational health and safety; he is a Certified Industrial Hygienist. The audit procedures included in-process monitoring of study activities for conformance to the study protocol and examining the records and supporting data. The dates and locations of each audit visit are listed in Table A.1 with the phase of the study examined.

AUDITORS' SUMMARY COMMENTS

Written reports for each inspection were provided to the Director of Science at the Health Effects Institute, who was responsible for transmitting 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 final report or the data contained therein. The Investigators' Final Report appears to be an accurate representation of the study.



Martin Anderson CIH, CSP
Auditor

Table A.1 Auditor's Responsibilities

Date, Site, and Investigator Visited	Phase of Study Audited and Audit Tasks
5/18/98 Health and Safety Executive Laboratory, Sheffield, United Kingdom Neil Plant	Analysis of air samples Review Good Laboratory Practices (GLPs); data management and protection; and record keeping
5/20/98 BD monomer production and polymerization facilities, Czech Republic Radim Šrám	Study population site location Review air sample collection procedures; equipment calibration procedures; biological sample collection, labeling, and storage procedures; and record keeping
5/21/98 Laboratory for Genetic Ecotoxicolgy, Prague, Czech Republic Radim Šrám	SCEs Review sample receiving and blinding procedures; sample preparation, storage, and protection procedures; GLPs; and record keeping
11/9/98 Leiden University Medical Center, Leiden, The Netherlands Ad Tatus	*HPRT* mutations in T lymphocytes Review GLPs; sample receiving, storage, and protection procedures; data storage and backup procedures
11/10/98 Shell International, Amsterdam, The Netherlands Nico van Sittert	BD urinary metabolites BD-Hb adducts Review GLPs; sample receiving, storage, and protection procedures; data storage and backup procedures
12/8/98 University of North Carolina, Chapel Hill, North Carolina James Swenberg	BD-Hb adducts Review GLPs; sample receiving, storage, and protection procedures; data storage and backup procedures
12/9/98 University of Texas Medical Branch, Galveston, Texas Jonathan Ward	*HPRT* mutations in T lymphocytes Review GLPs; sample receiving, storage, and protection procedures; data storage and backup procedures
12/10/98 Shell Westhollow Technical Center, Houston, Texas Alex Evans	BD urinary metabolites Review GLPs; sample receiving, storage, and protection procedures; data storage and backup procedures
12/11/98 Lovelace Respiratory Research Institute, Albuquerque, New Mexico Rogene Henderson	BD urinary metabolites Review GLPs; sample receiving, storage, and protection procedures; data storage and backup procedures
12/14/99 University of Vermont, Burlington, Vermont Richard Albertini	*HPRT* gene mutations Biostatistical analysis of data Review conformance to the study protocol (ie, record keeping, data management and protection, checking data for accuracy)
7/19/01 University of Vermont, Burlington, Vermont Richard Albertini	Final Report Audit

ABOUT THE PRIMARY AUTHORS

Richard J Albertini received an MD in 1963 and a PhD in medical genetics in 1972, both from the University of Wisconsin. He is a research professor in the Department of Pathology, an adjunct professor of microbiology and molecular genetics, and an emeritus professor of medicine at the University of Vermont in Burlington. Dr Albertini created the University of Vermont Genetic Toxicology Laboratory in 1985 and remains its director. In 2000, he became vice president for research at BioMosaics, Inc, a biotechnology company associated with the University of Vermont, with a focus on diseases of the immune system and cancer. Dr Albertini has been involved in studies of environmental mutagenicity and carcinogenicity for more than three decades, focusing on the causes and consequences of human somatic mutations. He developed the assay used to detect *HPRT* mutations in humans and has remained closely associated with research in this area. He works broadly in the area of human biomarkers and in the emerging area of molecular epidemiology.

Radim J Šrám received an MD from the Charles University in Prague in 1964, a PhD from the Czechoslovak Academy of Sciences in 1970, and a DSc from the Charles University in 1978. Since 1991 he has headed the Laboratory of Genetic Ecotoxicology, an organization of the Regional Institute of Hygiene of Central Bohemia and the Institute of Experimental Medicine Academy of Sciences, Czech Republic. He has worked in the field of genetic toxicology and molecular epidemiology for more than 30 years, studying genotoxicity in mammalian models and human populations. In the 1980s, he developed the network of genotoxic laboratories of the Czech Hygienic Service. In 1990, he established the International Teplice Program (concerned with the impact of environmental pollution on the health of human populations), which involves the US EPA and various laboratories in Europe.

Pamela M Vacek is a biostatistician in the Department of Medical Biostatistics and research assistant professor in the Pathology Department, University of Vermont College of Medicine in Burlington. She received an MS in biostatistics from the University of Vermont in 1976 and a PhD in occupational epidemiology from the University of London in 1994. Dr Vacek's main research interest is cancer epidemiology focused on quantifying occupational and environmental exposure to carcinogens, assessing risk factors for breast cancer, and evaluating the efficacy of breast-cancer screening.

Jeremiah Lynch received a BS in civil engineering from Rensselaer Polytechnic Institute in 1953 and, later, an MS in sanitary engineering from Massachusetts Institute of Technology and an MS in industrial hygiene from Harvard University. While working at US NIOSH, he became director of physical science and engineering. In 1976, Mr Lynch was hired by Exxon Chemical Company as manager of industrial hygiene and later became senior environmental health scientist. In 1996 he retired from Exxon and currently works on several research projects and as a lecturer.

Janice A Nicklas received a BS in biology from the California Institute of Technology in 1975 and a PhD in genetics from Princeton University in 1981. She is a research associate professor of medicine at the University of Vermont in Burlington and is currently on leave to the Vermont State Forensic Laboratory. Her research interests include molecular analysis of somatic mutations and differences in susceptibility to DNA damage after exposure to carcinogens and mutagens. Currently, she is developing methods to quantify human DNA using real-time polymerase chain reactions. Dr Nicklas is also interested in the effects of hemochromatosis mutations on DNA repair, DNA damage, and cancer.

Nico J van Sittert received his PhD in biochemistry in 1972. He worked in clinical chemistry for two years, and thereafter, for 25 years, in occupational health and toxicology for Shell. He was manager of the Biomedical Laboratory in Rotterdam (The Netherlands) from 1980 to 1993, and of the Molecular Toxicology Laboratory in Sittingbourne (UK) and Amsterdam (The Netherlands) from 1994 to 2000. He has published extensively in the fields of industrial or molecular toxicology. Currently he works as an independent consultant in toxicology.

Peter J Boogaard received an MS in analytical chemistry in 1984, a PharmD in 1985, and a PhD in toxicology in 1990 from Leiden University. He has held various senior positions with Shell; most recently he joined Shell International BV in The Hague as senior consultant in human toxicology at the Department of Health Services. His research interests have included mercapturic acids and hemoglobin adducts as biomarkers of exposure to industrial chemicals; and physiologically based pharmacokinetic modeling to assess the in vitro toxicology of BD for improved human risk assessment, (potential) industrial carcinogens (including inhalation, transdermal, and in vitro metabolism and toxicology), and BD biomarkers.

Rogene Henderson received a PhD in chemistry from the University of Texas in Austin in 1960. After a six-year stay at the University of Arkansas School of Medicine as a research associate, she joined the staff of the Lovelace Medical Foundation. In 1970, she transferred to the Inhalation Toxicology Research Institute, where she became supervisor of the Chemistry and Biochemical Toxicology Group. She is currently a senior scientist and deputy director of the National Environmental Respiratory Center at the Lovelace Respiratory Research Institute, Albuquerque NM. Her major research interests are in the use of bronchoalveolar lavage fluid analyses to detect and characterize the pulmonary response to inhaled toxins and the use of biomarkers to assess the risk from exposure to xenobiotics.

James A Swenberg received a DVM from the University of Minnesota in 1966 and a PhD in veterinary pathology from Ohio State University in 1970. He is currently professor of environmental sciences and engineering, pathology, and nutrition in the Schools of Public Health and Medicine at the University of North Carolina at Chapel Hill and director of the curriculum of toxicology. His research interests include understanding critical mechanisms in carcinogenesis, with emphasis on the role of DNA damage and repair, molecular epidemiology, and improving the scientific basis of risk assessment.

Ad D Tates received a PhD from Leiden University in 1971. Until he retired in 2000, he had been a researcher, teacher, and associate professor in the Department of Radiation Genetics and Chemical Mutagenesis since that laboratory was founded in 1959. His early research focused on studies of induction and repair of genetic damage from radiation and chemicals in germ cells of *Drosophila melanogaster*. More recently, his research dealt with (1) induction of chromosomal damage by chemical and physical agents in mammalian cells in vivo and in vitro, (2) development of methods for automated detection of micronuclei in human lymphocytes exposed to clastogenic agents, (3) biomonitoring human populations exposed to mutagenic agents by means of detecting chromosomal damage and *HPRT* point mutations, and (4) development and utilization of techniques to detect *HPRT* mutations in mice, rats, and humans.

Jonathan B Ward Jr received a PhD in microbiology from Cornell University in 1972. He is a professor and director of the Division of Environmental Toxicology, Department of Preventive Medicine and Community Health, at the University of Texas Medical Branch at Galveston. Dr Ward is a genetic toxicologist interested in the effects of mutagenic agents in exposed human populations and in mechanisms of genotoxicity in animal and cell-culture systems. For several years, his research has focused on the mutagenic effects of occupational exposure to BD by using the autoradiographic and cloning versions of the *HPRT* mutant lymphocyte assay to study populations of workers and laboratory mice. His most recent studies have addressed the role of *epoxide hydrolase* polymorphisms in human sensitivity to butadiene.

Michael Wright received a Graduate Membership qualification of the Royal Society of Chemistry by examination (equivalent to a BS in chemistry), from the University of Salford, England, in 1972. In 1990 he was awarded an MS by the University of Surrey, England. He is currently a senior scientist at the UK Health and Safety Laboratory in Sheffield, where he advises regulatory authorities on the monitoring implications of changes in OELs for chemical agents. His research interests include the theory of nonideal diffusive air sampling devices and the effectiveness of proficiency testing schemes for assessing chemical agents in the workplace.

COLLABORATING INSTITUTIONS

Burlington, Vermont, USA

Genetic Toxicology Laboratory, the Biometry Facility, and the Department of Medical Biostatistics, University of Vermont

Richard J Albertini, Pamela M Vacek, and Janice A Nicklas

with the assistance of Linda S Sullivan and Ingeborg Gobel

- The central study office: maintained codes and records from all participating laboratories; coordinated communication among all investigators; prepared interim and final reports
- Identified *HPRT* mutational spectra
- Carried out procedures for genotyping subjects
- Scored autoradiographic slides for *HPRT* variant frequencies for verification
- Conducted data analyses

Prague, the Czech Republic

Laboratory of Genetic Ecotoxicology, Regional Institute of Hygiene of Central Bohemia

Radim J Šrám

with the assistance of Blanka Binkova, Petra Musilová, Gabriela Rajská, Pavel Rössner, and Jirí Rubeš

- Assessed exposure levels; gathered subjects' questionnaires; collected and processed biological samples and distributed them to other laboratories
- Conducted cytogenetic analyses

Amsterdam, The Netherlands

Molecular Toxicology Laboratory, Shell International Chemicals, BV

Nico J van Sittert and Peter J Boogaard

with the assistance of Hendricus JJJ Megens, and Alex Evans (at Shell Westhollow Technical Center, Houston Texas, USA)

- Analyzed urinary M2 and M1 metabolites
- Conducted HBVal Hb adducts assays

Chapel Hill, North Carolina, USA

Curriculum of Toxicology, University of North Carolina at Chapel Hill

James A Swenberg

with the assistance of Patricia B Upton and Asoka Ranasinghe

- Conducted THBVal Hb adduct assays

Leiden, The Netherlands

Department of Radiation Genetics and Chemical Mutagenesis, Leiden University

Ad D Tates

with the assistance of Frans A de Zwart and Ailko H Zwinderman

- Performed *HPRT* mutation assays with the cloning protocol

Galveston, Texas, USA

Division of Environmental Toxicology, University of Texas Medical Branch at Galveston

Jonathan B Ward Jr

with the assistance of Marinel M Ammenheuser and Judah I Rosenblatt

- Performed *HPRT* mutation assays with the autoradiographic protocol

Rumson, New Jersey, USA

Jeremiah Lynch, Private Consultant

- Designed the exposure assessment scheme
- Participated in designing the study protocols for subject selection, data gathering, and exposure assessment

Sheffield, United Kingdom

United Kingdom Health and Safety Laboratory

Michael Wright

with the assistance of Neil Plant

- Provided sorbent tubes and analyzed them for personal exposure and workplace area measurements of BD and VOCs

Albuquerque, New Mexico, USA

National Environmental Respiratory Center, Lovelace Respiratory Research Institute

Rogene F Henderson

with the assistance of Jennifer R Krone, Dean A Kracko, and Walter B Blackwell

- Conducted urinary metabolite analyses [data not reported]

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Albertini RJ, Šrám RJ, Vacek PM, Lynch J, Wright M, Nicklas JA, Boogaard PJ, Henderson RF, Swenberg JA, Tates AD, Ward JB Jr. 2001. Biomarkers for assessing occupational exposures to 1,3-butadiene. Chem Biol Interact 135–136:429–453.

Albertini RJ. 2002. Developing sustainable studies on environmental health. Mutat Res 480–481:317–331.

Albertini RJ. 2003. Mechanistic insights from biomarker studies: Somatic mutations and rodent/human comparisons following exposure to a potential carcinogen. IARC Sci Publ (in press).

Albertini RJ, Clewell H, Himmelstein MW, Morinello E, Olin S, Preston J, Scarano L, Smith M, Swenberg J, Tice R, Travis C. 2003. The use of non-tumor data in cancer risk assessment: Reflections on benzene, butadiene and vinyl chloride. ILSI Risk Science Institute, Working Group on the Use of Non-tumor Data in Cancer Risk Assessment. Regul Toxicol Pharmacol (in press).

ABBREVIATIONS AND OTHER TERMS

ADH	alcohol dehydrogenase
ANOVA	analysis of variance
BCS	bovine calf serum
BD	1,3-butadiene
BD-diol	1,2-dihydroxy-3-butene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
BDO-G	total of N7-(1-[hydroxymethyl]-2-propenyl)-guanine and N7-(2-hydroxy-3-butenyl)guanine; the hydroxybutenyl adducts at N7 of guanine
BEL	biological exposure limit
bp	base pair

BrdU	5-bromo-2′-deoxyuridine
cDNA	complementary DNA
CARB	California Air Resources Board
CE	cloning efficiency
CEFIC	European Chemical Industry Council
CI	confidence interval
CYP	*cytochrome P450* family of genes
CYP	cytochrome P450–related enzymes and proteins
CYP2E1	cytochrome P450 2E1
$[d_6]$-M2	deuterium-labeled isomeric mixture of M2
DAPI	4,6-diamidino-2-phenylindole
DBS	iron-supplemented defined BCS
DHHS	US Department of Health and Human Services
DHP-MA	*N*-acetyl-*S*-(2,3-dihydroxypropyl)-L-cysteine
DMSO	dimethylsulfoxide
dNTPs	deoxynucleoside triphosphates
ECs	evaluatable cells
EDTA	ethylenediaminetetraacetic acid
EH	epoxide hydrolase
EPA	US Environmental Protection Agency
$F_G/100$	genomic frequency of stable chromosomal translocations per 100 cells
FID	flame ionization detector
FISH	fluorescence in situ hybridization
GC	gas chromatography
GC-ECNCI-HRMS	gas chromatography with electron-capture negative chemical ionization and high-resolution mass spectrometry
GC-MS	gas chromatography with mass spectrometry
GC-MS/MS	gas chromatography with tandem mass spectrometry
GC-NCI-MS/MS	gas chromatography with negative chemical ionization and tandem mass spectrometry
GC-NECI-MS/MS	gas chromatography with negative-electron-capture ionization and tandem mass spectrometry
GLP	Good Laboratory Practices
GSH	glutathione
GST	glutathione *S*–transferase
GSTM1	*glutathione* S–*transferase M1* genotype
GSTT1	*glutathione* S–*transferase T1* genotype
Hb	hemoglobin
HBVal	isomeric mixture of *N*-(1-[hydroxymethyl]-2-propenyl)valine and *N*-(2-hydroxy-3-butenyl)valine
HCl	hydrochloric acid
HFCs	high frequency cells (cells with > 12 SCEs)
HPLC	high-performance liquid chromatography
HPRT	*hypoxanthine phosphoribosyltransferase* gene
HSE	Health and Safety Executive
$[^3H]$TdR	tritiated thymidine
IARC	International Agency for Research on Cancer
IISRP	International Institute for Synthetic Rubber Producers
IL-2	interleukin-2
IVS	intervening sequence (or intron)
KCl	potassium chloride
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LI	labeling index
LOD	limit of detection
M1	1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane
M2	isomeric mixture of 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene and 2-hydroxy-1-(*N*-acetylcysteinyl)-3-butene
MDHS	Methods for the Determination of Hazardous Substances
MF	mutant frequency
$MgCl_2$	magnesium chloride
MNCs	mononuclear cells
MRM	multiple-reaction monitoring
mRNA	messenger RNA
MS	mass spectrometry
N-1-THB-A	*N*-1-(2,3,4-trihydroxybutyl)adenine
$NaHCO_3$	sodium bicarbonate
$(NH_4)_2SO_4$	ammonium sulfate
NIOSH	US National Institute of Occupational Safety and Health
NTP	US National Toxicology Program

OEL	occupational exposure limit
OSHA	US Occupational Safety and Health Administration
PAHs	polycyclic aromatic hydrocarbons
PBLs	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFPITC	pentafluorophenylisothiocyanate
PFPTH	pentafluorophenylthiohydantoin
PTFE	polytetrafluoroethylene
PHA	phytohemagglutinin
ppb	parts per billion
ppm	parts per million
QC	quality control
r	Spearman correlation coefficient
R^2	coefficient of determination for multivariate analysis
RBCs	red blood cells
RT-PCR	reverse transcriptase–PCR
SBR	styrene-butadiene rubber
SCEs	sister chromatid exchanges
S/N	signal-to-noise ratio
Taq	*Thermus aquaticus*
TG	6-thioguanine
−TG	medium without the TG selection agent
+TG	medium with the TG selection agent
THB-G	N7-(2,3,4-trihydroxybutyl)guanine; trihydroxybutyl adducts at N7 of guanine
THBVal	*N*-(2,3,4-trihydroxybutyl)valine
TWA	time-weighted average
VF	variant frequency
V_{max}	maximal velocity of enzyme reaction
VOCs	volatile organic compounds
WASP	Workplace Analysis Scheme for Proficiency

ALTERNATE TERMS FOR BUTADIENE AND ITS METABOLITES AND ADDUCTS

BD	α,γ-butadiene
	biethylene
	bivinyl
	buta-1,3-diene
	divinyl
	erythrene
	pyrrolylene
	vinylethylene
	trans-butadiene
BDO	3,4-epoxy-1-butene (EB)
	butadiene monoepoxide (BMO or BDO)
BDO_2	1,2:3,4-diepoxybutane
	butadiene diepoxide (BDE)
	diepoxybutane (DEB)
BD-diol	3,4-dihydroxy-1-butene
BDO-diol	3,4-epoxy-1,2-butanediol (EBD)
	butadiene diol-epoxide
	butadiene monoepoxide-diol
HBVal	total of 1- and 2-monohydroxy-3-butenyl valine
M1	1,2-dihydroxybutyl mercapturic acid (DHB-MA)
	N-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine
M2	1,2-hydroxybutyl mercapturic acid (MHB-MA)
	1-(*N*-acetylcysteinyl)-2-hydroxy-butene
	isomeric mixture of the regio- and stereo-isomers (*R*)/(*S*)-*N*-acetyl-*S*-(1-[hydroxy-methyl]-2-propenyl)-L-cysteine + (*R*)/(*S*)-*N*-acetyl-*S*-(2-hydroxy-3-butenyl)-L-cysteine
	monohydroxy-3-butenyl mercapturic acid (MHB-MA)
	total 1- and 2-hydroxy-3-butenyl mercapturic acid (MHB-MA)

COMMENTARY
Health Review Committee

INTRODUCTION

1,3-Butadiene (BD)* is a four-carbon gaseous chemical synthesized for use in the manufacture of resins, plastics, and synthetic rubber. It is also produced by combustion; BD is present in cigarette smoke and in emissions from mobile and stationary sources.. Although the highest BD exposures of 0.022 to 660 mg/m^3 (0.01 to 299 parts per million [ppm]**) occur in industrial settings, ambient exposures of about 0.0022 to 0.022 mg/m^3 (0.001 to 0.01 ppm) may be of public health concern because BD is carcinogenic in rats and mice (reviewed by Himmelstein et al 1997). BD exposure levels and the evidence for BD carcinogenicity in humans has been reviewed by Melnick and Kohn (1995), the European Centre for Ecotoxicity and Toxicology of Chemicals (1997), Himmelstein and coworkers (1997), and the International Agency for Research on Cancer (IARC 1999) (also see the later Scientific Background section).

In 1994, HEI initiated a research program to address the health risks of ambient exposure to toxic air pollutants, including benzene and BD. The overall goal of this program was to gather information that would reduce uncertainties in evaluating the human health risks associated with exposure to low levels of toxic air pollutants from mobile sources. As part of this program, HEI has supported studies (1) to assess the mutagenic and carcinogenic effects of BD and its reactive metabolites on cells and in laboratory animals and (2) to develop biomarkers that could serve as measures of BD exposure (Health Effects Institute 2000). Such biomarkers need to be validated in human populations before they can be considered for use in full-scale epidemiologic studies.

In 1995, HEI issued Request for Qualifications 95-3, "Transitional Epidemiology Studies for Benzene or 1,3-Butadiene Biomarkers," which sought researchers with access to benzene-exposed or BD-exposed human populations. Applicants first provided HEI with descriptions and characterizations of the exposed and control populations that would be studied and the qualifications of the study team. In January 1996, several investigators with access to BD-exposed populations and several others with access to benzene-exposed populations were selected to participate in planning workshops with other scientists having expertise in biomarker evaluation for BD or benzene. The purpose of the workshops was to facilitate collaborations among the investigators with expertise in biomarker evaluation and scientists with access to exposed populations.

In the second stage of the RFQ process, investigators with access to exposed populations submitted full applications to HEI. Dr Richard Albertini at the University of Vermont and collaborators submitted a proposal entitled "Biomarker Responses in Butadiene-Exposed Czech Workers: A Transitional Epidemiologic Study." Dr Radim Šrám at the Laboratory of Genetic Ecotoxicology (Regional Institute of Hygiene of Central Bohemia) was a key investigator who provided contact with the BD-exposed workers. Albertini and collaborators in the United States, The Netherlands, the United Kingdom, and the Czech Republic proposed to validate specific biomarkers in the blood and urine from workers occupationally exposed to BD in the Czech Republic. If successful, this validation study would allow these biomarkers to serve as surrogate measures of BD exposure in epidemiologic studies.

Because the samples collected in the Czech Republic were to be sent to widely scattered laboratories, the investigators wisely proposed a pilot study (Phase I) to test the logistics of collecting and shipping samples. Phase I would also allow Albertini to further define the project plan, gather information necessary to plan the research phase (Phase II), bring together the coinvestigators and consultants to work on study design issues, and prepare standard operating procedures. The HEI Health Research Committee thought this was an effective and ambitious proposal whose major strengths were the scientific team and the use of an exposed population that had participated in earlier studies (see the Scientific Background section that follows). In addition, Albertini provided an excellent rationale for selecting the proposed biomarkers. The Committee viewed the Phase I pilot study as an important component and recommended that it be extended from the proposed 3 months to 6 months. They also concluded that Albertini needed to recruit a coinvestigator with extensive experience in the field of exposure assessment, a key component of the project plan. The Committee then recommended funding the proposed study.

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

**For ease of comparison with the Investigators' Report, all measurements for BD are given in mg/m^3 followed by ppm in parentheses regardless of how the levels of BD were reported in the published articles being discussed; the conversion factor used was ppm $\times$ 2.21 = mg/m^3 (as specified in the Investigator's Report).

In April 1997, Albertini submitted a revised budget to reflect a lengthened Phase I and added a consultant (Mr Jeremiah Lynch) with extensive experience in the analysis of industrial exposures to BD. Lynch evaluated the exposure conditions and records at the facilities and concluded that it would be feasible to conduct a concurrent exposure assessment and to evaluate previously documented exposures.[†]

SCIENTIFIC BACKGROUND

Epidemiologic studies have suggested that occupational exposure of workers to BD is associated with an increased incidence of cancer of the lymphatic system (lymphosarcoma) and of the organs and systems in the body that produce blood cells (leukemia) (Himmelstein et al 1997; US Environmental Protection Agency [EPA] 1998; IARC 1999). Increases in these two types of cancer were noted in workers exposed to BD in two styrene-butadiene rubber (SBR) plants (Meinhardt et al 1982; Matanoski and Schwartz 1987; Matanoski et al 1990). More recent and comprehensive studies of the same worker group indicated an increased risk of leukemia (but not other types of cancers) in workers with a long duration of employment in the SBR industry (Delzell et al 1996; Macaluso et al 1996; Sathiakumar et al 1998). However, the interpretation of these studies is somewhat controversial. Even though BD was the major chemical to which these workers had been exposed and the statistical analyses controlled for exposure to styrene, the subjects had been exposed to several other chemicals as well. As a result, it was unclear whether the increased incidence of cancers was due to BD or the other chemicals present in the workplace.

In addition, studies of workers in the BD monomer industry, where occupational exposure is restricted to BD, showed no relation between BD exposure and leukemia (Divine 1990; Divine et al 1993; Divine and Hartman 1996). Instead, the studies showed a small increase in the number of deaths from two forms of sarcoma (tumors that usually arise from connective tissue), lymphosarcoma and reticulosarcoma, in workers who began their employment before World War II.

These cohort studies have been difficult to interpret (EPA 1998; IARC 1999) because of (1) the differences in the types of cancers reported for the BD monomer and the SBR industrial groups, (2) possible confounding exposures of SBR workers to other chemicals, (3) changes in the definition and classification of the lymphohematopoietic cancers, and (4) uncertainty about the relative importance of short-term, high-level exposures versus long-term, low-level exposures in causing health effects. (HEI has continued to investigate these issues; especially, a study by Dr Elizabeth Delzell is reanalyzing mortality among North American synthetic rubber industry workers, a major occupational cohort on which the US EPA has based its decisions about the health risks associated with butadiene exposure.)

Government and international agencies have assessed BD's carcinogenicity in humans. In 1999, IARC concluded that the evidence available for human carcinogenicity was limited because it was based on the increased incidence of leukemias reported in one large study and because two or more studies of adequate statistical power had not yet reported consistent results. Therefore, IARC retained its earlier designation of BD as a probable human carcinogen (IARC 1999). The US National Toxicology Program upgraded its designation of BD to known human carcinogen (US Department of Health and Human Services 2000). In its health risk assessment of BD in 2002, the EPA concluded that BD is carcinogenic to humans by inhalation (EPA 2002). The US National Institute for Occupational Safety and Health (US Department of Health and Human Services 1991) and the US Occupational Safety and Health Administration (1996) concluded that BD is at least potentially carcinogenic to humans.

In those situations where epidemiologic data were considered to be limited, quantitative risk assessments of BD exposure have relied on information from animal studies of BD metabolism or carcinogenicity. However, laboratory studies of rodents present their own challenges for data interpretation because, although BD is converted to the same metabolites in rats and mice, the rate at which these metabolites are formed differs between the two species; the result is that mice are much more sensitive to the carcinogenic effects of BD than rats (Himmelstein et al 1997). Humans also convert BD to the same metabolites found in these rodents, but because of the differences between rats and mice, researchers are unsure which rodent species should serve as a model for human BD metabolism and risk.

BD is converted in the body via a complex metabolic pathway; a simplified version is diagrammed in Figure 1 of the Investigators' Report. BD is not itself a direct-acting mutagen or carcinogen. When taken up in the body, predominantly by inhalation, it is converted by enzymes in

[†]Dr Albertini's study, *Biomarker Responses in Butadiene-Exposed Czech Workers: A Transitional Epidemiologic Study*, began in November 1997. The draft Investigators' Report was received in December 1999. After review by a Panel of the HEI Health Review Committee in February 2000, a revised report was received and accepted for publication in February 2001. During the review process, the HEI Health Review Committee and the investigators had an opportunity to exchange comments and to clarify issues in both the Investigators' Report and the Review Committee's Commentary.

Total expenditures for this study were $1,154,369. Funding was provided by HEI's core sponsors (the U.S. Environmental Protection Agency and the automotive industry) and the American Chemistry Council, the European Chemical Industry Council, and the International Institute of Synthetic Rubber Producers.

many tissues to epoxide metabolites that have the potential to produce harmful effects (referred to as reactive metabolites). Other enzymatic reactions can detoxify the epoxides to metabolites that are excreted in the urine. BD is oxidized by cytochrome P450–monooxygenase to the reactive monoepoxide intermediate, 1,2-epoxy-3-butene (BDO), which can be further metabolized to another reactive epoxide, 1,2,3,4-diepoxybutane (BDO_2), or to 1,2-dihydroxy-3-butene (BD-diol). Both of these can in turn be metabolized to another reactive epoxide, 1,2-dihydroxy-3,4-epoxybutane (BDO-diol). One or more of the BDO, BDO_2, and BDO-diol metabolites can (1) react with macromolecules such as DNA or proteins, (2) be detoxified by epoxide hydrolase (EH) or glutathione *S*–transferase (GST) to produce metabolites excreted in the urine, or (3) be converted to other metabolites by alternative pathways (not shown in Figure 1).

WHAT IS A TRANSITIONAL EPIDEMIOLOGIC STUDY?

Transitional epidemiologic studies are designed to bridge the gap between purely laboratory investigations and purely epidemiologic field studies. A critical component of any transitional epidemiologic study designed to validate a biomarker as a reliable measure of either dose or effect is an accurate measurement of exposure. The intent of transitional studies is to lay the groundwork for a biomarker to be used in full-scale epidemiologic studies by assessing (1) its variability within and among subjects, (2) the feasibility of its use in field studies, and (3) any confounding or effect-modifying factors. Thus, transitional epidemiologic studies are designed to validate a biomarker but are usually not designed to have enough statistical power to draw conclusions about health outcomes.

BIOMARKERS AND MOLECULAR EPIDEMIOLOGY

Biomarkers appear at various early stages in the pathway leading from exposure, to a carcinogenic response, to clinical disease. They permit the detection of recent exposures in humans, facilitate internal dosimetry, and may identify effects of exposure that could be relevant to the mechanism of a disease process (National Research Council 1987). The use of biomarkers can alleviate some of the weaknesses of traditional epidemiologic studies in occupational cancer research, such as the need to successfully link past exposures to more recent disease and the need to study large populations because of the low incidence of specific cancers. Because biomarkers of exposure and effect appear relatively frequently in exposed populations, significant increases in biomarker levels can be observed in small populations (Ward et al 1996). Biomarkers can also serve as a basis for comparisons among species or among levels of exposures when comparing dose-response relations. For example, a relevant biomarker along the causal pathway to cancer could be compared in rats and mice exposed to different concentrations of a chemical. If this biomarker were also found and validated in occupationally exposed individuals, it might help clarify the relation between exposure level and cancer risk in humans (Collins 1998). In addition, genotypes (individuals' genetic compositions) could be compared in people with and without different types of cancers to see if certain enzymes that affect carcinogenic processes are present or absent in their genetic coding (see the later section Biomarkers Chosen for This Study / Factors That May Affect Susceptibility).

Molecular epidemiologic approaches involving biomarkers have great potential in several areas of cancer research: investigating the etiology of the disease, monitoring cancer risk in people exposed to occupational or environmental carcinogens, studying factors that protect against cancer, and assessing intrinsic factors that might predispose an individual to cancer (Collins 1998). Some biomarkers are specific to a certain chemical and some are not. The biomarkers most commonly employed in molecular epidemiologic cancer research include:

- DNA damage in lymphocytes (breaks in DNA, bulky adducts produced by a chemical binding to DNA bases);
- adducts produced by a chemical binding to proteins such as those found in blood;
- urinary metabolites or products of DNA damage excreted in urine;
- Micronuclei and chromosomal alterations (including translocations);
- sister chromatid exchanges (SCEs);
- mutations (altered sequence of DNA bases) in reporter genes; and
- differential expression of those enzymes involved in the metabolism of carcinogens that may affect an individual's susceptibility.

BIOMARKERS CHOSEN FOR THIS STUDY

The investigators focused on two classes of biomarkers.

- *Biomarkers of Exposure*: Metabolites of BD released in urine (biomarkers of recent exposure); and hemoglobin (Hb) adducts (biomarkers of somewhat longer exposure). (The investigators planned to measure DNA adducts also, but the assay that had been developed for animal exposures to high levels of BD [Blair et al 2000] was not sensitive enough to assess human exposures to lower occupational levels.)

- *Biomarkers of Effect*: Frequency of mutations at the *HPRT* gene in T lymphocytes; differences in the *HPRT* mutational spectrum; and, in whole blood lymphocytes, chromosomal changes such as SCEs, aberrations, and translocations.

In addition, the investigators looked at factors that may affect susceptibility to carcinogens, such as genetic polymorphisms in *GSTM1*, *GSTT1*, *cytochrome P450 2E1* (*CYP2E1*), *EH*, and *alcohol dehydrogenase* (*ADH*).

Biomarkers of Exposure

Urinary Metabolites The major metabolites of BDO found in urine are 1,2-dihydroxy-4-(*N*-acetylcysteinyl)-butane, referred to as M1, and an isomeric mixture of 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene and 2-hydroxy-1-(*N*-acetylcysteinyl)-3-butene, referred to as M2. M1 is a mercapturic acid formed by hydrolysis of BDO to BD-diol, which is then conjugated with glutathione (GSH). M2 is an isomeric mixture of mercapturic acids formed by direct conjugation of BDO with GSH. Monkeys and humans produce primarily M1 because of their naturally high levels of EH (the enzyme that catalyzes hydrolysis). In contrast, rats, mice, and hamsters produce more M2 because of lower EH levels (Sabourin et al 1992; Bechtold et al 1994). Legator and coworkers (1993) reported that workers exposed to BD (2.2–6.6 mg/m^3; 1–3 ppm) and unexposed control subjects had only M1 in their urine, although the M1 levels were higher in the BD-exposed workers. Thus, M1 is a more sensitive measure than M2 for monitoring human exposure to BD.

Hb Adducts Blood concentrations of reactive BD-epoxide intermediates can be measured in laboratory animals after high exposures to BD, but this approach to dosimetry is not possible in humans exposed to the low levels typically found in BD manufacturing industries. BD reaction products bound to blood proteins, such as Hb adducts, can be used to detect and quantify exposure to chemicals that are themselves reactive or have been transformed in the body to reactive intermediates. The analysis of protein adducts is highly sensitive and specific to the chemical (Skipper and Tannenbaum 1990; Osterman-Golkar et al 1996). Unlike DNA adducts, which can be removed by DNA repair processes and lost to further analysis, Hb adducts are not removed and typically accumulate over the lifespan of the red blood cell (120 days on average). Thus, these adducts can be measured after exposure to even low levels of BD and are potentially useful biomarkers of human exposure. Osterman-Golkar and associates (1993) pointed out additional advantages of measuring Hb adducts as markers of BD exposure: (1) blood samples are easy to obtain and contain large amounts of Hb; (2) Hb adducts can improve exposure assessments by providing data about exposure over time; and (3) Hb adducts provide a measure of the dose of reactive chemicals in both laboratory animals and humans, thereby eliminating some of the difficulties in extrapolating from high to low doses across species.

Osterman-Golkar and coworkers (1991) identified BDO adducts in Hb obtained from the blood of rats exposed to 553 to 2210 mg/m^3 (250 to 1000 ppm) BD 6 hours a day, 5 days a week, for 2 weeks. Adduct levels in blood samples obtained after 12 days of exposure increased linearly with BD exposure and ranged from 0.5 to 3.0 nmol/g Hb. The adducts differed in their three-dimensional forms (called diastereomers) and in the portion of the BDO molecule that reacts with valine (called positional isomers); collectively, these adducts are termed hydroxybutenyl valine (HBVal) adducts. HBVal adducts were also identified in the blood of workers that were exposed occupationally to BD (Osterman-Golkar et al 1993, 1996; van Sittert and van Vliet 1994). Pérez and coworkers (1997) identified trihydroxybutyl valine (THBVal) adducts, which exist as four diastereomers (but not as positional isomers), in blood from rats and humans exposed to BD. THBVal can be formed by reaction of BDO_2 or BDO-diol with the *N*-terminal valine of globin. Although Pérez and colleagues (1997) concluded that the contribution of each pathway was unknown, their results suggested that BDO-diol is more active than BDO_2 in producing THBVal adducts and that BDO-diol may therefore play a major role in BD-induced toxicity. Swenberg and coworkers (2000) identified both HBVal and THBVal adducts in the blood of rats, mice, and humans exposed to BD.

Sorsa and coworkers (1996) evaluated the effects of BD exposure on HBVal adduct levels in exposed workers. In a Portuguese BD manufacturing plant, air samples were collected over a full workshift by personal monitors in the workers' breathing zones; 30% contained more than 0.44 mg/m^3 BD (0.2 ppm BD). At monomer and SBR production facilities in the Czech Republic (the same ones studied by Albertini and colleagues in the present research), Sorsa and associates found typical BD concentrations ranging from 0.44 to 4.4 mg/m^3 (0.2 to 2 ppm); 10% of the samples exceeded 22 mg/m^3 (10 ppm), and in a few instances, exposures over 1100 mg/m^3 (498 ppm) were recorded. In each facility, higher levels of Hb adducts were detected in the blood of exposed workers compared with control maintenance workers. Hb adduct levels were higher in the blood of workers exposed in the SBR plant compared with workers in the monomer facility (Sorsa et al 1996).

Biomarkers of Effect

HPRT *Mutations* The development of mutations in cells is a biomarker of effect from exposure to a mutagen. Although the *HPRT* gene is not involved in carcinogenesis, mutations in this gene may indicate that mutations have also occurred in genes that affect carcinogenesis. For this reason, *HPRT* is called a reporter gene. An advantage of studying *HPRT* mutations is that the assays are simple, straightforward, and require only cells cultured from a blood sample. Two assays were used in the study by Albertini and colleagues: the cloning assay, which identifies mutant cells; and the autoradiographic assay, which identifies variant cells. (These cells are termed variants because the autoradiographic method, discussed below, cannot confirm the genetic change in the mutant cells. However, the ability of variant cells to synthesize DNA under selective culture conditions implies that they have mutated.) These assays are described in the later section Technical Evaluation / Methods.

Ward and colleagues (1996) performed two studies (8 months apart) on the effects of BD exposure on small groups of workers in a BD monomer plant and a third study at an SBR plant, both in Texas. In the first monomer plant study, exposures were estimated from a survey conducted by the company 3 to 9 months before blood and urine samples were collected; the survey yielded an estimated average of 2.2 mg/m^3 (1 ppm) for all exposed workers studied. In the second study, exposures were determined from 8-hour air samples collected from workers' personal breathing zones. The BD levels were 0.66, 0.46, and 0.26 mg/m^3 [0.30, 0.21, and 0.12 ppm], respectively, in areas of high, intermediate, and low exposure. At the SBR plant (the third study), exposures were determined by passive samplers worn by workers in their breathing zones during an 8-hour shift on the same day biological samples were collected. (Passive samplers depend on air naturally diffusing toward the detection matrix, whereas larger, active samplers use a small pump to deliver air.) Half of the breathing-zone samples exceeded 0.55 mg/m^3 (0.25 ppm) BD, and of these, slightly more than half exceeded 2.2 mg/m^3 (1 ppm). In all three studies, the *HPRT* variant frequencies (VFs) of cells identified by autoradiography were significantly higher in blood from workers in the highest-exposure areas than from workers in lower-exposure areas or from unexposed subjects. However, the authors of papers on these studies suggest caution in interpreting the relation between air concentrations of BD and *HPRT* VFs because BD exposure levels were measured in different ways and over different time periods in the three studies. In the first two studies of the Texas workers (both at the BD monomer plant), Ward and colleagues (1996) also measured the concentrations of urinary metabolites (discussed earlier as biomarkers of exposure). In the first study, the concentration of M1 increased significantly in BD-exposed workers compared with control subjects and this increase correlated with increases in VF (a biomarker of effect). In the second study, M1 levels did not increase significantly in exposed workers, probably because the plant had undergone a modernization program that reduced BD exposure. (M1 levels in workers were not reported for the third study, which had been conducted at the SBR plant.)

Studies of larger groups of workers in the same Texas SBR plant were conducted a year later by the same laboratory, also using the autoradiographic assay. Ward and colleagues (2001) compared *HPRT* VFs and urinary M1 metabolites in 37 workers exposed to high (mean 7.03 mg/m^3 [3.18 ppm]) and low (mean below the limit of detection of 0.55 mg/m^3 [0.25 ppm]) BD concentrations. Levels of both biomarkers were three times higher in the more-exposed workers than in the less-exposed workers. VFs, M1 levels, and BD exposure concentrations were all correlated. Ward and coworkers (2001) then conducted a follow-up study at the same SBR plant. Although the highest BD levels to which workers were exposed (3.71 mg/m^3 [1.68 ppm]) were lower than in the earlier study, the *HPRT* VFs were still threefold higher in the more-exposed group than in the less-exposed group (mean exposure level below the limit of detection). M1 levels were also still higher in the more-exposed group, albeit to a lesser degree than in the earlier study (Ward et al 2001).

After the study described in this Research Report was completed, Ward and colleagues (Ammenheuser et al 2001) reported another study of workers in the Texas SBR plant: 24 workers exposed to an average level of 3.26 mg/m^3 (1.48 ppm) BD and 25 workers exposed to approximately 0.33 mg/m^3 (0.15 ppm) BD; each subject's exposure was measured during one 8-hour workshift. (For comparison, the current research evaluated 34 SBR [polymerization] workers, although they were not divided into high- and low-exposure groups.) That Ward study again found that lymphocyte *HPRT* VFs and urinary M1 levels were threefold higher in the high-exposure group than in the low-exposure group. BD and styrene levels both were measured by passive samplers. The mean molar ratio of BD to styrene was 27.92; therefore, BD exposure levels were substantially greater than styrene exposure levels. Furthermore, VFs, M1 levels, and BD exposure concentrations were all correlated. The authors concluded that the correlations among BD exposure and the biomarkers of both exposure (M1) and effect (VFs) suggest that occupational exposure to BD levels in the range of 2.2 mg/m^3 to 6.6 mg/m^3 (1–3 ppm) may be associated with adverse biological effects.

In contrast to the elevated *HPRT* VFs reported in all the studies of Texas workers described above (all of which used the autoradiographic assay to identify cell variants; Ward et al 1996, 2001; Ammenheuser et al 2001), two studies that used the cloning assay to identify cells with *HPRT* mutations found no effect of BD on mutant frequencies (MFs) in lymphocytes from exposed workers compared with those from unexposed workers (Tates et al 1996; Hayes et al 1996). In 1993 and 1994, Tates and coworkers (1996) examined lymphocytes from 19 exposed and 19 unexposed workers in the BD monomer plant in the Czech Republic (the same one studied by Albertini and colleagues in the current research); workers had been exposed to a mean BD level of 3.87 mg/m^3 (1.75 ppm). The lack of BD effect on *HPRT* MFs was shown when 1993 and 1994 blood samples were pooled and analyzed and also when 1994 samples were analyzed alone. Hayes and coworkers (1996) also found no effect of BD on *HPRT* MFs in 41 workers (compared with 38 unexposed workers) exposed to BD at a median level of 4.4 mg/m^3 (2 ppm) in a Chinese polymer plant. They suggested that their results differed from those of Ward and coworkers (1996) because (1) differences in cell culture methods may have led to measuring mutant or variant cells in different lymphocyte subpopulations; and (2) the small number of subjects in all the studies may have affected statistical variability. However, a different study from Ward's laboratory (Ma et al 2000) also used the cloning assay to identify *HPRT* MFs: 10 workers at the Texas SBR facility exposed to a median BD level of 1.90 mg/m^3 (0.86 ppm) had significantly elevated MFs compared with results from 11 unexposed control workers.

At this time, the reasons for such contradictory results between studies evaluating VFs and studies evaluating MFs in different worker populations are still unexplained. Both the Tates and Ward groups participated in the study of Czech workers described in this Research Report. In their section of the Investigators' Report (Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Discussion), they concluded that "We cannot explain why it was not possible to demonstrate induction of *HPRT* mutations in lymphocytes from BD-exposed workers in the Czech Republic [in the present study] and in China, whereas it was possible to detect a significant increase in the MFs for exposed workers when the cloning assay was used for BD-exposed workers in Texas."

Mutational Spectra The sequence of purine and pyrimidine bases in a gene is altered when a gene undergoes mutation and the pattern of these changes is referred to as the mutational spectrum. Changes in DNA sequence can be identified by the type of mutation (eg, point mutations [alterations at a specific base pair within a gene], or insertions or deletions of one or more bases), the location of the specific mutation in the chain of DNA bases, and the frequency of the specific mutation. The pattern of these changes is often specific for a given chemical; thus, the mutational spectrum produced can be considered a fingerprint of exposure to that chemical. For example, inhalation of BD causes an increase in point mutations primarily occurring at A•T base pairs in mice (Cochrane and Skopek 1994; Sisk et al 1994; Recio and Meyer 1995; Recio et al 1996); these increases are often concentrated at specific areas of the DNA sequence and are called mutational hotspots.

SCEs and Chromosomal Aberrations Sister chromatids are copies of a chromosome produced during its replication cycle. Sister chromatid exchange is the symmetric exchange of DNA segments between the sister chromatids of a duplicated chromosome (Norppa 1997). The exchanges most likely reflect a disruption of the normal replication process by the presence of abnormal DNA. Thus, SCEs are a sensitive indicator that cells have been exposed to a DNA-damaging agent (Collins 1998). Nevertheless, we do not understand what molecular mechanisms are responsible for SCEs, what biological role they have, or how they relate to chromosomal aberrations.

Chromosomal aberrations also may occur after cells are exposed to DNA-damaging agents. Such aberrations include breaks, deletions, translocations (a segment of a chromosome moves to a different site on the same chromosome or to a site on another chromosome), and duplications (Collins 1998).

Factors That May Affect Susceptibility

Individuals may differ in their susceptibility to cancer due to any number of factors (eg, health, nutritional status, environmental exposures, gender); genetic composition also may be important (reviewed by Hirvonen 1999). Genes for some enzymes are polymorphic, meaning the gene can have several different forms that are commonly found in the general population; each form, or allele, has a variation in its sequence of bases. Some of the alleles code for enzymes with altered activity levels. In some instances, the gene may not produce an active enzyme, in which case it is called a null allele. Genes that might influence cancer susceptibility include those that encode the enzymes that metabolize carcinogens. For example, certain BD metabolites have been implicated in BD-induced cancer in animals; modifications in the genes that encode the enzymes involved in BD metabolism may alter the enzymes' activities. Thus, depending on their genetic composition, people

may to a greater or lesser degree convert BD to the metabolites thought to be involved in cancer induction. Knowing which alleles an individual carries may help identify their expected response to BD exposure. For this reason, subjects in the current study were tested for genotypes for several polymorphic genes (*CYP2E1*, *EH*, *ADH*, and *GST*) to determine if these might be genetic factors that would change a person's susceptibility to BD exposure or in some way influence the expression of the biomarkers related to BD exposure.

GENETIC POLYMORPHISMS AND CYTOGENETIC DAMAGE

Genetic traits that confer increased susceptibility to cytogenetic damage from reactive BD metabolites could be important risk factors for cancer in BD-exposed populations (Wiencke et al 1995). Differences in biological effects that are influenced by variations in genotype can be evaluated by cytogenetic methods that analyze chromosomal aberrations and SCEs. The few studies of genetic polymorphisms and the frequency of in vivo or in vitro cytogenetic damage to human cells have been performed almost exclusively on peripheral blood lymphocytes; these cells are easily available and easily cultured by following well-established techniques (Norppa 1997).

Because the GST enzyme has such an important role in metabolizing reactive epoxides, we focus the discussion here on the genetic variations of the *GST* gene. Among the white population, approximately 50% possess the *GSTM1*-null genotype and 10% to 20% possess the *GSTT1*-null genotype (Bernardini et al 1996). These genotypes have been studied in association with several of the reactive BD metabolites.

Some researchers have targeted their studies on three reactive BD metabolites (BDO, BDO-diol, and BDO_2), active and null alleles of *GSTT1* and *GSTM1*, and the possible outcomes of SCEs and chromosomal aberrations. First, Uusküla and colleagues (1995) and Bernardini and coworkers (1998) measured BDO-induced SCEs in cultured human lymphocytes containing the active and null alleles of *GSTM1* and *GSTT1*; the lymphocytes with either null allele had an increased level of SCEs compared with the alleles with normal enzyme activities. Thus, both null alleles have the potential to increase the genotoxic effects of BDO. Second, in a study with BDO-diol (Bernardini et al 1996), the levels of human lymphocyte SCEs induced by two concentrations of BDO-diol (250 or 500 μM) in culture were dose-dependent, but were independent of the presence or absence of the *GSTT1* or *GSTM1* genotypes. Therefore, neither the GSTT1 nor the GSTM1 enzyme appeared to be involved in the detoxification of BDO-diol. Third, Landi and coworkers (1998) demonstrated that exposure to BDO_2 increased the frequency of SCEs and the number of cells with chromosomal aberrations in human lymphocytes with the *GSTT1*-null genotype compared with lymphocytes with the active *GSTT1* allele; the *GSTM1* genotype did not affect these endpoints. Thus, in these published studies, BDO-induced SCEs were influenced by both *GSTT1*-null and *GSTM1*-null alleles, BDO-diol–induced SCEs were influenced by neither, and BDO_2-induced SCEs were influenced only by the *GSTT1*-null allele.

Lymphocytes from humans not known to have been exposed to BD or other genotoxins varied in their sensitivity to BDO_2 (Wiencke et al 1995). Blood samples were taken from 72 subjects and the lymphocytes exposed to BDO_2. Lymphocytes from 12 subjects showed elevated levels of SCEs compared with those from the other 60 subjects. Of the 12 lymphocyte samples with elevated SCEs, 6 individuals carried a deletion in the *GSTT1* gene that was not evident in any of the subjects whose lymphocytes did not respond to BDO_2. This concordance between the deletion and high SCE induction was incomplete, however, because half of the individuals whose lymphocytes responded to BDO_2 did not carry the gene deletion. Thus, polymorphism in the *GSTT1* gene may affect a person's response to BDO_2 exposure.

Šrám and coworkers (1998) studied lymphocytes from monomer production workers exposed to BD (0.024 to 23.0 mg/m^3 [0.011 to 10.4 ppm]) in the same facility studied in the current research. Those lymphocytes showed a significantly increased percentage of chromosomal aberrations, frequency of SCEs, and percentage of cells with a high number of SCEs compared with lymphocytes from unexposed workers. In the same worker population, Sorsa and coworkers (1994) had initially found no changes in chromosomal aberrations or SCEs. However, when the data were reanalyzed, they found a significantly higher frequency of chromosomal aberrations in lymphocytes from BD-exposed workers who had the *GSTT1*-null allele compared with those from exposed workers who had the *GSTT1*-active allele. The presence or absence of the *GSTM1*-active allele had no effect on chromosomal aberrations.

TECHNICAL EVALUATION

STUDY DESIGN

This transitional epidemiologic study was designed to evaluate the sensitivity with which several biomarkers reflected quantified, low-level, occupational exposures to BD. Overall, the study was well conceived and well planned.

The subjects were employed by a single company that operates a BD monomer production facility and a polymerization facility near Prague, the Czech Republic. The entire staff (24 men) of the BD monomer production facility and about half of the work force (34 men) in the BD polymerization facility participated in the study. Most of those who did not participate were employed by an independent contractor. Only a few workers directly employed by the factory declined to participate. The control cohort comprised 25 male administrative workers employed by the same company who were selected, first, because they were males and, second, because they were willing to participate. No attempt was made to randomly select control subjects; this could have led to possible selection bias.

The demographic summary in Tables 5 and 6 in the Investigators' Report indicates that the three exposure groups were fairly well balanced in terms of age, length of employment by the company, time of residence in the district, and personal smoking status. For exposure to passive smoke, however, all monomer production workers reported such exposure and this differed significantly from the number of administrative control subjects (72%) and polymerization workers (74%) who also reported exposure. The participants also differed substantially in several other factors: (1) the administrative control subjects had more years of education; (2) the monomer production workers may have been somewhat healthier than the administrative control subjects and polymerization workers in that they reported a lower rate of infectious illness; and (3) the administrative control subjects had the highest vaccination rate, the least frequent use of antibiotics (of 14 medications charted), and more frequent vitamin and vegetable intake.

The study was designed to be largely cross-sectional; biological samples for biomarkers were collected within a short time frame near or at the end of a 60-day exposure assessment period, during which exposure measurements had been taken at several intervals. This design accommodated the lengthy half-lives of some of the biomarkers and preserved the biomarkers that would reflect short-term exposure. This approach, although critical for biomarker development and validation, has not been followed in many epidemiologic studies to date. It is a major strength of this study. The specific timing of the 60-day exposure assessment period was offset by 1 or 2 weeks for different groups of workers so that biological fluids would not need to be collected from all workers on the same days. Blood samples were obtained on the last day of exposure assessment and urine samples were obtained before and after workshifts on the last 3 days. The first urine sample was collected after a 2-day rest period from work, which the investigators thought would be sufficient time to eliminate the BD urinary metabolites before the first before-shift urine sample was collected.

Each worker's personal exposure to BD was measured on several occasions by small sampler tubes attached to his clothes. Other samplers were analyzed for toluene, styrene, and benzene. (The exposure protocol and samplers are described in more detail in the Methods section below.) The Health and Safety Executive Laboratory in Sheffield, United Kingdom, was contracted to measure the BD, toluene, styrene, and benzene levels collected by the samplers. Personal monitoring was supplemented by measuring workplace area concentrations of each compound at several locations.

The study was conducted in a blinded fashion: Each subject was assigned a coded identification number at the study's onset; this code and others assigned to biological samples were not revealed to any investigator in any laboratory until all analyses in that laboratory were complete. Furthermore, an elaborate chain of custody procedure was followed to track all samples through collection, processing, shipping, and analyzing. After biological samples were collected at the workers' facility, they were transported on ice immediately to Šrám's laboratory in Prague and processed within hours of collection. Each sample or cell fraction was assigned a unique identifier that related it to the sample donor. Samples were then stored at −70°C until they were shipped to the collaborating laboratories.

BD biomarkers, factors that may affect susceptibility, and exposure monitoring tubes were investigated at the following institutions [**bold type** indicates the place names used to identify different laboratories throughout this Report]:

- Urinary M2 and M1 metabolites and HBVal Hb adducts by Dr Nico van Sittert, Dr Peter Boogaard, and colleagues at the Molecular Toxicology Laboratory, Shell International Chemicals, BV, **Amsterdam**, The Netherlands.
- THBVal Hb adducts by Dr James Swenberg and associates at the Curriculum of Toxicology, University of North Carolina at Chapel Hill, **Chapel Hill**, North Carolina, USA.
- *HPRT* mutant cells (via the cloning assay) by Dr Ad Tates and colleagues at the Department of Radiation Genetics and Chemical Mutagenesis, Leiden University, **Leiden**, The Netherlands; *HPRT* mutational spectra by Dr Jan Nicklas and Dr Richard Albertini and associates at the Genetic Toxicology Laboratory, University of Vermont, **Burlington**, Vermont, USA.
- *HPRT* variant cells (via the autoradiographic assay) by Dr Jonathan Ward Jr and colleagues at the Division of Environmental Toxicology, University of Texas

Medical Branch at Galveston, **Galveston**, Texas, USA; independent slide review of the autoradiographic assays by Dr Albertini and associates at the Biometry Facility, University of Vermont, **Burlington**, Vermont, USA.

- *GSTM1* and *GSTT1* genotypes, SCEs, and chromosomal aberrations by Dr Radim Šrám and colleagues at the Laboratory of Genetic Ecotoxicology, Regional Institute of Hygiene of Central Bohemia, **Prague**, Czech Republic.
- *CYP2E1*, *ADH*, and *EH* genotypes by Dr Albertini and colleagues at the Genetic Toxicology Laboratory, University of Vermont, **Burlington**, Vermont, USA; genotype assays at the Vermont Cancer Center DNA Analysis Facility, **Burlington**, Vermont, USA.
- Sorbent tubes for personal exposure and workplace area measurements of BD and volatile organic compounds (VOCs) by Michael Wright and associates at the United Kingdom Health and Safety Laboratory, **Sheffield**, United Kingdom.
- Data analyses by Dr Albertini, Dr Pamela Vacek, and associates at the Department of Medical Biostatistics, University of Vermont, **Burlington**, Vermont, USA.

In most cases, the logistics of sample collection, processing, and shipping worked well. *GSTM1* and *GSTT1* genotypes and Hb adducts were evaluated in all 83 study participants. In the case of urinary biomarkers, the first day's collection of after-work samples was complete; however, a before-work sample from one polymerization worker could not be included because of insufficient volume. In addition, some difficulties arose in preparing samples for some of the *HPRT* mutation assays: For example, although 95% of the samples were analyzed by the cloning method, only 59% of the samples were analyzed by the autoradiographic assay because the remaining samples did not have the number of cells needed for reliable evaluation. The low number of evaluatable cells may have been due to the condition of the cells after shipment. In addition, some sorbent tubes used for exposure monitoring were mislabeled.

The investigators largely met their experimental objectives. They were able to evaluate the usefulness of the chosen biomarkers for assessing BD exposures by relating the biomarkers to measured exposures. The effort expended in Phase I of this project paved the way for the successful completion of Phase II. Although the conduct of the study deviated somewhat from the proposed plan and some difficulties arose in preparing some samples, the investigative team formulated and implemented a solid study design. The integration of a comprehensive exposure assessment with a series of logical biomarker analyses was unprecedented for such a complex, international study.

METHODS

The methods used in this biomarker study with multiple endpoints were state of the art. Below is a brief overview of some of the various methods. Greater detail can be found in the matching sections of the Investigators' Report.

Exposure Assessment

Passive samplers attached to the workers' clothes during 8-hour shifts measured their personal exposures to BD; each measurement was recorded as a TWA for the work shift. Measurements were taken at time intervals that were relevant to the biomarkers being evaluated. For example, during a 60-day exposure assessment period for each exposed subject, about six to eight measurements were irregularly spaced before biological samples were collected; this allowed investigators to measure biomarkers that would develop or persist over weeks or months (*HPRT* mutations, Hb adducts, chromosomal translocations). On each of the last 3 days of the exposure assessment period, personal exposures were again measured to compare with biomarkers that would reflect recent exposures (urinary metabolites, SCEs, chromosomal aberrations).

VOCs (benzene, toluene, and styrene) were also measured via passive samplers on one day for some subjects from each exposed group. Seven measurements were made at irregular intervals designed to cover all shifts of the control subjects. Personal BD and VOC measurements were obtained on the same day for all except five of the subjects who wore VOC samplers (two administrative control subjects, two monomer production workers, and one polymerization worker). The protocol for these VOC measurements was designed to determine if any coexposures might track with BD and confound the interpretation of exposure results. A more extensive assessment of VOCs was not thought to be warranted because of the cost, the availability of personal monitors, and the fact that VOCs were not the primary focus of this study.

Active samplers measured workplace area levels of BD and VOCs as 15-minute concentrations at selected locations. These measurements were made primarily to assess whether the personal monitoring results (recorded as workshift time-weighted averages [TWAs]) were reasonable determinants of the workers' exposures. Thus, concentrations from 15 minutes of sampling (rather than from a full workshift) were deemed sufficient to evaluate workplace levels of the agents of interest.

Exposure samplers were all analyzed at Sheffield. The gases collected on the sampler matrix were desorbed by heat, separated by gas chromatography, and quantified.

Biomarkers of Exposure

Urinary Metabolites M2 and M1 were extracted from urine and then derivatized, first with methanol and then with pentafluorobenzoylchloride. The derivatives were separated and quantified by gas chromatography with negative-electron-capture ionization and tandem mass spectrometry. Because metabolite levels may be concentrated or diluted depending on the rate of urine production, the investigators normalized their data by expressing them in values related to units of urinary creatinine.

Hb Adducts: HBVal and THBVal For HBVal, globin was prepared from red blood cell Hb by the Edman degradation technique (details provided in the Investigators' Report) and reacted with pentafluorophenylisothiocyanate to form a pentafluorophenylthiohydantoin (PFPTH) derivative of the *N*-terminal valine of globin with its BD adducts (a mixture of the C_1 and C_2 positional isomers). This PFPTH derivative was detected by gas chromatography with negative-ionization tandem mass spectrometry and multiple-reaction monitoring.

For THBVal, globin was isolated from Hb and the *N*-terminal valine of globin with its trihydroxybutyl adduct was prepared by a modification of the Edman degradation technique. THBVal was acetylated and detected by gas chromatography–high-resolution mass spectrometry in the electron-capture negative-chemical-ionization mode.

Biomarkers of Effect

HPRT *Mutants and Variants* The product of the *HPRT* gene is the enzyme hypoxanthine guanine phosphoribosyltransferase, which metabolizes the purine bases hypoxanthine and guanine. This enzyme can also metabolize a purine base analog, 6-thioguanine (TG), to an intermediate that is toxic to normal lymphocytes. Thus, one way to differentiate normal lymphocytes from those that have mutated at the *HPRT* locus is to determine whether they can grow in the presence of TG. Normal blood lymphocytes, which can produce an active HPRT enzyme, are killed in cultures to which TG is added. In contrast, lymphocytes with mutations in the *HPRT* gene do not produce an active HPRT enzyme and therefore divide in the presence of TG.

In the *HPRT* cloning assay, lymphocytes are cultured for 10 to 14 days in plates with 96 small, individual wells in the presence of TG and then evaluated by light microscopy for the presence or absence of viable cell colonies; the viable colonies contain mutant cells. MF is calculated by comparing the fraction of wells with viable cell colonies versus those without.

The autoradiographic assay does not require cell division. In this procedure, lymphocytes are incubated in culture medium with TG for 24 hours. Radioactive thymidine is then added. The cells' uptake of radioactivity serves as a measure of DNA replication because, when it is taken up by the cells, the radioactivity becomes part of the newly synthesized DNA. Cell nuclei are then isolated. Nuclei with radioactive DNA, evident by the darkening of a photographic emulsion, are identified as variant. (Because the cells die during the process of detecting their radioactivity, the assay cannot confirm mutations.)

HPRT *Mutational Spectra* A total of 441 *HPRT* mutant cells, isolated with the cloning assay, were allowed to multiply in culture to expand the number of cells and allow isolation of mRNA and DNA. For a variety of technological or methodological reasons, the molecular analysis of point mutations by applying reverse transcriptase–polymerase chain reaction (PCR) techniques could be performed on only 24% (108) of the 441 isolated mutant clones. Likewise, multiplex-PCR, used to detect mutations associated with large deletions, could be carried out successfully for 38% (166) of the 441 isolated mutants. The mutants that could be analyzed at the molecular level were equally distributed over the three study groups.

Cytogenetic Changes: SCEs and Chromosomal Aberrations The investigators estimated and compared three groups of cytogenetic endpoints using different assays and procedures. First, they used conventional chromosome staining to detect breaks, deletions, and rearrangements. Second, the investigators used a technique called fluorescence in situ hybridization (FISH), which detects specific chromosomes and chromosomal changes (Trask et al 1993). In the FISH technique, a probe (a sequence of DNA that is synthesized to bind specifically to a complementary strand of chromosomal DNA) is coupled to a fluorescent chemical to allow the bound probe/DNA complex to be detected by fluorescence microscopy. With this method, the investigators identified chromosomal aberrations, several types of translocations, and conjunctions (the latter defined as a connection or link among colors between chromosomes in the FISH PAINT process). Third, they identified SCEs in lymphocytes that had been cultured in the presence of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) and then stained. BrdU is incorporated into newly synthesized DNA. Exchanges between sister chromatids result in a pattern

of stained and nonstained regions that reflect the exchanges. This process allowed the investigators to determine the number of SCEs/cell; cells with a high frequency of SCEs (eg, more than 12) were called high frequency cells (HFCs).

Factors That May Affect Susceptibility: Metabolic Genotypes

Genotyping for *GSTM1* and *GSTT1* was performed on mononuclear cells from fresh blood samples. Changes in the base sequences of the genes coding for *GSTM1* and *GSTT1* were detected by ultraviolet illumination of the gel and recorded by photography.

The investigators used DNA isolated from lymphocytes obtained during the *HPRT* cloning assay to identify *ADH2*, *ADH3*, *EH*, and *CYP2E1* genotypes. In general, the investigators used methods similar to those described above for *GSTM1* and *GSTT1*.

STATISTICAL ANALYSES

The following text summarizes some of the statistical approaches used by Albertini and colleagues. Specific statistical methods for analyzing subject information, exposures, and each biomarker are described in the Investigators' Report in the section Study Design: Subject Selection, Sample Acquisition and Handling, and Statistical Methods / Data Processing and Statistical Analysis.

Multiple exposure measurements for each worker are expected to show variability. To characterize this variability, the investigators used variance component analysis that employed a random-effects model with day of exposure and subject as the random effects. Separate analyses were performed for monomer production and polymerization workers.

Differences among the three exposure groups for all exposure metrics and biomarkers were determined by one-way analysis of variance (ANOVA), using pairwise multiple comparisons, and by the nonparametric Kruskal-Wallis test, using Bonferoni-adjusted Mann-Whitney tests for pairwise comparisons.

The investigators assessed bivariate relations among different biomarkers and among biomarkers and exposure variables by using Spearman correlation coefficients to test whether larger values of one variable tended to be paired with larger values of the other variable, regardless of the functional form of the relation. They also determined Pearson correlation coefficients to assess the linearity of the relations. Regression analysis was used to quantify the relation of urinary metabolites with mean BD exposure and Hb adducts with mean BD exposure, and to evaluate associations among biomarkers. Multiple regression analyses were conducted to examine the effects of exposure (as quantified by mean BD exposure, by mean BD exposure 70 to 50 days before blood sampling, or by concentration of Hb adducts) on the cytogenetic and *HPRT* biomarkers after adjusting for age and smoking status. Exposure group was included in some models to identify group differences that were independent of BD exposure. The investigators also used multiple regression analyses to examine whether genotype modified the relations among BD exposure levels and biomarkers.

RESULTS AND INTERPRETATIONS

Exposure Assessment

The primary goal of the exposure assessment protocol was to provide a summary measure of the exposure each monomer production and polymerization worker received over the 60-day exposure assessment period that could be correlated with the levels of biomarkers of exposure (urinary metabolites and hemoglobin adducts). All exposure values were recorded as TWAs for data collected throughout an 8-hour workshift. The mean BD exposure for the control group was 0.026 mg/m^3 (0.012 ppm) based on 28 individual TWAs (range: 0.002 to 0.125 mg/m^3 [0.001 to 0.057 ppm]); for the monomer group, it was 0.643 mg/m^3 (0.291 ppm) based on 217 individual TWAs (range: below 0.0018 [the limit of detection] to 20 mg/m^3 [0.0008 to 9 ppm]); and for the polymer group, it was 1.760 mg/m^3 (0.796 ppm) based on 319 individual TWAs (range: 0.002 to 39.030 mg/m^3 [0.001 to 17.661 ppm]). Most of the variability in measurements for the two exposed groups was due to a small number (approximately 10%) of high measurements: 90% of the measurements for the monomer production workers were less than 1.883 mg/m^3 (0.852 ppm), and for the polymerization workers they were less than 3.985 mg/m^3 (1.803 ppm).

Workplace area measurements (from 15-minute pumped samples) yielded mean BD concentrations of 0.043 mg/m^3 (0.019 ppm) for the administration area (based on 18 samples), 0.316 mg/m^3 (0.143 ppm) for the monomer unit (based on 60 samples), and 0.892 mg/m^3 (0.375 ppm) for the polymerization unit (based on 89 samples). All differences between pairs of exposure areas (administration, monomer production unit, polymerization unit) were statistically significant.

ANOVA confirmed that the main source of measurement variability was day-to-day variation within subjects. As explained by the investigators, day-to-day variation in BD exposures illustrates the importance of obtaining multiple measurements of exposure for each worker. Such

comprehensive exposure assessment was a major strength of this study.

Mean personal exposures (also TWAs) to benzene for the control, monomer, and polymer groups were 0.030 mg/m^3 (0.009 ppm), 0.039 mg/m^3 (0.012 ppm), and 0.064 mg/m^3 (0.020 ppm), respectively. Mean personal exposures to toluene were 0.295 mg/m^3 (0.078 ppm), 0.162 mg/m^3, (0.043 ppm), and 0.681 mg/m^3 (0.181 ppm), for the same exposure groups. No differences among exposure groups for these two VOCs were statistically significant. Mean personal exposures to styrene for the control, monomer, and polymer groups were 0.118 mg/m^3 (0.027 ppm), 0.147 mg/m^3 (0.035 ppm), and 2.121 mg/m^3 (0.498 ppm), respectively. As expected because workers in the polymerization process are exposed to styrene, the mean TWA styrene exposure for the polymer group was significantly different from the means for both the monomer and control groups; the difference between the means for the monomer and control groups was not statistically significant. Note that the one-time VOC measurements were taken at different times: late April (control group), mid March (monomer group), and mid-to-late April (polymer group).

Workplace area measurements of benzene, toluene, and styrene were consistent with those obtained by personal monitoring but, as with the BD measurements, they were lower. Benzene exposures were moderately correlated with BD exposures. Styrene exposures were more highly correlated with BD exposures than was benzene. Toluene exposures were unrelated to BD concentrations.

The investigators considered the mean BD exposures in this study to be moderate, which in their opinion indicates a well-run facility. The group means were within the range of those reported in other similar studies (Sorsa et al 1994, 1996; Au et al 1995; Tates et al 1996; Ward et al 1996; Hayes et al 2000) in which monomer production workers were, on average, exposed to lower concentrations of BD than polymerization workers.

Biomarkers of Exposure

Urinary Metabolites Urinary metabolites proved to be valuable biomarkers of BD exposure.

As noted earlier, the investigators collected urine samples on the last 3 days of each worker's exposure assessment period. They first analyzed day-1 samples and these results are presented in the following text. Day-2 and day-3 samples were stored for later analysis. When they wanted to analyze day-3 samples, the Amsterdam laboratory had closed and analysis at another laboratory was unsuccessful.

The limit of detection for M1 was 5 µg/L and for M2 was 0.1 µg/L. The after-work mean M1 concentrations were 353, 764, and 4647 µg/L for the control, monomer, and polymer groups, respectively. The differences between the monomer and control groups and between the polymer group and control or monomer groups were statistically significant. Because humans detoxify BD predominantly by way of the EH pathway (see Figure 1 in the Investigators' Report), M1 levels were much higher than M2 levels. However, statistically significant differences in after-work samples of M2 were also evident (1.70, 9.44, and 120.17 µg/L for the control, monomer, and polymer groups, respectively). The net (after-work minus before-work) M1 levels showed similar, statistically significant relations among the three groups. Although the monomer and polymer groups both had significantly higher net M2 levels than the control group, the difference between the two exposed groups was not significant. Each subject's after-work and net M2 and M1 levels were highly correlated with his mean exposure over the entire assessment period. Although BD exposure levels could not be measured for all workers on each day of urine collection, after-work urinary metabolite levels were highly correlated with same-day BD exposure levels measured in subsets of workers from each exposure group. In addition, net M2 and net M1 levels were correlated with same-day BD exposures in the subsets, although these correlations were lower than the correlations between after-work levels and same-day BD. Statistical analyses indicated that neither *GSTM1* nor *GSTT1* polymorphisms affected levels of M2 or M1 in any exposure group.

As discussed in the earlier section Technical Evaluation / Study Design, the investigators believed that a two-day rest period from work would allow enough time to sufficiently eliminate residual M2 and M1 metabolites from the workers' urine. This belief was based on previous findings that the half-lives of the mercapturates of many industrial chemicals (eg, allyl chloride, 1,3-dichloropropene, epichlorohydrin, ethylene oxide, benzene) in urine are short (van Welie et al 1991; Boogaard and Van Sittert 1995, 1996; de Rooij et al 1997a,b). An unexpected finding was that M2 and M1 were present at elevated levels in before-shift urine samples from polymerization workers. Both the M2 and M1 levels were higher than those found in samples from both other exposure groups, but only the difference in M2 levels was statistically significant. Thus, the elimination of M2 was incomplete even 48 hours after the most recent occupational BD exposure.

The investigators propose that M1 (the major BD metabolite in human urine) in samples from administrative control subjects may have arisen from sources other than BD. Ward and coworkers (1994) also reported that subjects who did not work with BD excreted M1. However, the exposure assessment data presented above indicate

that the administrative control subjects experienced low but measurable BD exposures and thus cannot be considered completely unexposed. Therefore, M1 derived from endogenous or exogenous BD or from other sources may have made small contributions to the BD-induced M1 levels detected in each worker group in this study.

Hb Adducts Hb adducts proved to be even stronger biomarkers of BD exposure than urinary metabolites.

The limit of detection for HBVal in human blood was 0.1 pmol/g globin. The mean HBVal adduct concentrations were 0.224, 0.466, and 2.230 pmol/g globin for the control, monomer, and polymer groups, respectively. All differences between pairs of group means were statistically significant and the magnitude of differences paralleled those in the BD exposure levels of the three groups. The presence of Hb adducts in the administrative control subjects is not completely understood, although adduct levels were higher in smokers than in nonsmokers in this group and cigarette smoke contains BD. Mean HBVal levels (overall or within each group) were not associated with genetic variations at any of the susceptibility loci evaluated.

The limit of detection for THBVal in human blood was approximately 1 pmol/g globin. The mean THBVal adduct concentrations were 94.77, 178.73, and 716.70 pmol/g globin for the control, monomer, and polymer groups, respectively. All differences between pairs of group means were statistically significant and the THBVal adduct concentrations paralleled the BD exposure levels of the three groups. As was the case with HBVal adducts, smokers in the control group had higher levels of THBVal than nonsmokers. Genotype did not significantly affect mean THBVal levels.

Concentrations of HBVal and THBVal in blood were highly and significantly correlated within each exposure group. THBVal levels were 600-fold, 540-fold, and 368-fold higher than HBVal levels in control, monomer, and polymer groups, respectively. The difference in levels between the polymer group and each of the other two exposure groups was statistically significant. Therefore, both HBVal and THBVal appear to be useful biomarkers of BD exposure.

In concordance with their conclusions about the appearance of the urinary M1 metabolite in the control group, the presence of relatively high levels of THBVal in the control group suggests that it may arise not only in response to BD exposure but also from other sources. Swenberg and coworkers (2000) reported that blood samples from volunteers who do and do not smoke contained detectable levels of THBVal (40.2 pmol/g globin and 36.3 pmol/g globin, respectively). The detection of THBVal in Hb from smokers is not surprising. Its presence in nonsmokers suggests that it may be an endogenous adduct produced from compounds other than BD or from other sources of BD. Swenberg and coworkers (2000) cite their detection of THBVal in rodents, calves, dogs, horses, and squirrel monkeys. However, as discussed earlier, the administrative control subjects in the current study were exposed to low levels of BD and thus cannot be considered completely unexposed. Thus, as with the levels of urinary metabolites, endogenous sources of BD or other compounds may have supplemented the adduct levels in each group.

Biomarkers of Effect

In contrast to the positive responses of exposed workers (compared with administrative control subjects) who demonstrated an increased production of the biomarkers of exposure (urinary metabolites and Hb adducts), the studies on biomarkers of effect (reflected in gene mutations, mutational spectra, and cytogenetic effects) were uniformly negative. The small number of subjects available for the study and the fact that exposure levels were lower than initially expected limited the statistical power for analyzing some mutational endpoints.

HPRT *Mutant Cells Assessed via the Cloning Assay* Group mean MFs were 13.01×10^{-6}, 9.24×10^{-6}, and 18.83×10^{-6} for the control, monomer, and polymer groups, respectively. The differences in MFs between the pairs of exposure groups were not statistically significant, whether analyses were performed with or without an outlier data point in the polymer group. The fact that the MF for the control group was higher than that for the monomer group, although not statistically significant, agreed with the results of an earlier study of workers in this same Czech monomer production facility (Tates et al 1996); in that study, the MFs for the control group and the monomer workers were 13.93×10^{-6} and 10.10×10^{-6}, respectively. The cloning protocol differed slightly between the two studies but the assays were performed in the same laboratory and mainly by the same staff. These two data sets are in good agreement even though the control subjects in the 1996 study were workers from a heat production unit of the monomer plant rather than administrative workers, as in the current study.

In contrast to the differences between the monomer and control groups in the current study (described above), MFs in the polymer group were elevated, although not to statistical significance, compared with the control group. The investigators note that approximately 60 subjects per exposure group would have been required to demonstrate a statistically significant increase of 45% (1.45-fold) in

polymerization workers compared with control subjects. Because only 34 polymerization workers were available for this study, it did not have the statistical power to detect increases in MF as small as 45%.

To compare these data with the results from the autoradiographic assay (described later in this section), the MF calculations were repeated, this time excluding 20 samples with fewer than 400,000 clonable cells (corresponding to the lower limit of evaluatable cells used in the autoradiographic assay). The differences between pairs of groups decreased (MFs were 12.43×10^{-6}, 10.84×10^{-6}, and 12.77×10^{-6} for the control, monomer, and polymer groups, respectively) and still lacked statistical significance. Analysis of all three exposure groups indicated no statistically significant association between MFs and mean BD exposure, or between MFs and smoking or genotype. The investigators suggest that the number of workers was too few to detect small differences in MFs among exposure groups. The negative findings of the cloning assay are consistent with those of Tates and coworkers (1996) and Hayes and colleagues (2000) on Czech and Chinese BD workers but are in contrast to the results of the cloning assays on Texas workers reported by Ma and coworkers (2000).

HPRT *Variant Cells Assessed via the Autoradiographic Assay* Mean VFs were 10.75×10^{-6}, 5.73×10^{-6}, and 6.48×10^{-6} for the control, monomer, and polymer groups, respectively. Individual BD exposure levels were not correlated with individual VFs within any exposure group. The control group's mean VF was significantly higher than the mean VFs for both the monomer and polymer groups.

The administrative control subjects in this study had unusually high VFs compared with control workers in the Texas studies (Ward et al 1996, 2001; Ammenheuser et al 2001) and the current results are inconsistent with the results in those studies. The investigators discuss possible reasons for the high VF levels in the administrative control subjects in the section Biomarkers of Effect: Assays of *HPRT* Mutations in Human Lymphocytes / Discussion / The Autoradiographic Assay / Factors That Could Affect the Results of *HPRT* Assays.

The investigators point out that, for the nonsmokers of this study's control group, VFs were more than fivefold higher than the mean VF for historical control samples collected in the United States (Ammenheuser et al 1997). In fact, the VFs for smokers and nonsmokers in the control group of this study showed little effect of smoking. In the monomer group, nonsmokers had lower VFs than smokers; whereas in the polymer group, nonsmokers had higher VFs than smokers. Measurement of cotinine levels was not a part of the study design; therefore the investigators could not verify smoking behaviors reported by the workers.

The investigators state that evaluating autoradiographic slides by light microscopy involves a certain amount of subjective judgment among individual researchers identifying variant cells. The possibility of introducing an error by this procedure was controlled for in two ways. First, the slides were coded such that the researchers did not know to which subject or exposure group the slides belonged. Second, after the slides were evaluated at Galveston, they were reevaluated at Burlington; the two evaluations yielded similar results.

Mutational Spectra The mutational spectra of control and BD-exposed workers revealed no striking differences. This result was not surprising because BD-exposed workers did not show an increase in MF. However, it was important to compare mutational spectra because a small number of changes in DNA that are specific to BD exposure could have occurred in the absence of a measurable increase in MF. Although exposure to BD did not significantly affect the spectrum of mutations, as compared with control administrative workers, a few novel mutations were identified. These data will be of interest to investigators studying the molecular mechanisms of *HPRT* mutagenesis.

Cytogenetic Analyses The most important result of this component of the study was the lack of differences between control and BD-exposed workers in SCEs and in chromosomal aberrations measured by either the conventional method or by FISH.

Cytogenetic changes and genetic polymorphisms were associated in some ways. For example, statistical analysis showed a lower frequency of chromosomal aberrations resulting from BD exposure associated with one of the two EH genotypes evaluated. In addition, the percentage of aberrant cells and the number of DNA breaks per cell were lower in cells from workers with one of the two *ADH2* alleles evaluated. However, statistical analysis indicated that these changes were not related to BD exposure.

Given that the mean VF for the control group was higher than those for both the monomer and polymer groups (and higher than the Texas control subjects in a study by Ward and colleagues [1996]), it is not surprising that chromosomal aberrations in the control group would also be higher than historical control subjects as determined by the conventional method and by FISH techniques (Rubeš et al 1998; P Rössner, personal communication of unpublished results, 2000). The investigators point out that their historical control values have been obtained from various sources in the Czech Republic and may not be strictly comparable to values from industry control workers.

Factors That May Affect Susceptibility

Variations in genotype at polymorphic loci that may affect susceptibility to BD exposure were unrelated to BD levels in this study and had little or no quantitative influence (or effect) on biomarkers of exposure or effect. Some specific genotypes were associated with quantitative differences in dose-response relations of urinary metabolites or Hb adducts, although none indicated a heightened susceptibility to BD.

Correlations Among Biomarkers

Correlations Among the Biomarkers of Exposure Before-work (82 study participants) and after-work (all 83 study participants) urinary M2 and M1 values were positively and significantly correlated with HBVal Hb adduct concentrations. HBVal adduct concentrations also correlated significantly and positively with net (after-work minus before-work) urinary M2 and M1 metabolites, but the correlations were lower than the correlations between after-work M2 or M1 and HBVal.

As with HBVal, THBVal Hb adduct concentrations were also positively correlated with before-work and after-work urinary M2 and M1 values and, with the exception of the before-work M1 concentration, the correlations were statistically significant. THBVal adducts also were positively and significantly correlated with net M2 and M1 concentrations, but again the correlations were lower than the correlations between after-work M2 or M1 and THBVal.

Correlations Among the Biomarkers of Exposure and the Biomarkers of Effect No positive correlations were reported between any pairs of these two types of biomarkers for any of the exposure groups. Negative and statistically significant correlations were reported between *HPRT* VF levels and increases in each of the urinary metabolites for all of the exposure groups. Correlations between *HPRT* VF levels and each of the Hb adducts also were negative for each of the exposure groups, but only those involving THBVal were statistically significant. No statistically significant correlations were found for any of the exposure groups between (1) *HPRT* MF levels and any of the four biomarkers of exposure (M2, M1, HBVal, or THBVal), or (2) between any of the cytogenetic endpoints (determined by the conventional method or by FISH) and any of the biomarkers of exposure.

Correlations Among the Biomarkers of Effect *HPRT* mutation levels, expressed as either MFs or VFs, did not correlate with levels of cytogenetic biomarkers. Therefore, the investigators concluded that the biomarkers of genotoxic effects, whether mutational or chromosomal changes, were not only unrelated to BD exposure but were also unrelated to one another.

DISCUSSION OF MUTATION FINDINGS

The primary purpose of this study was to validate biomarkers as possible surrogate measures of actual exposure in a human population. None of the biomarkers was evaluated as a possible indicator or predictor of health outcomes. A unique feature of the study design is that it brought together the investigators of earlier major mutation analyses (Tates [Leiden; Czech BD workers] and Ward [Galveston; Texas BD workers]) to conduct parallel analyses in one population.

The current study did not detect significant differences in mutation levels among exposure groups. The results of the current study (see the earlier section Technical Evaluation / Results and Interpretations / Biomarkers of Effect / *HPRT* Mutant Cells Assessed via the Cloning Assay) both confirmed and are at odds with earlier studies of subjects in this and other workplaces. In the earlier section Scientific Background / Biomarkers Chosen for This Study / Biomarkers of Effect / *HPRT* Mutations, we discuss the unexplained differences in the MF results obtained by the cloning assay using lymphocytes from workers in both the Czech Republic and in China (in which both populations showed no increase in MF compared with control workers; Tates et al 1996; Hayes et al 1996) versus lymphocytes from workers in Texas (in which MFs were higher than those in control subjects; Ma et al 2000).

The results of the current study's analysis of variant levels (see the earlier section Technical Evaluation / Results and Interpretations / Biomarkers of Effect / *HPRT* Mutant Cells Assessed via the Autoradiographic Assay) also differ with those previously reported by researchers in the Galveston laboratory where the autoradiographic assay for VFs was performed. Those studies of Texas workers showed that workers exposed to higher levels of BD had higher VFs than workers exposed to lower BD levels and unexposed control subjects (Ward et al 1996, 2001; Ammenheuser et al 2001). A complication of the VF component of the current study, but not of earlier studies by Ward and coworkers', was the unexpectedly higher levels of VFs in the Czech control group than in the monomer and polymer groups. In comparison, the Leiden investigators using the cloning assay, also found higher MFs in the Czech control group than in monomer group both in this and in an earlier study (Tates et al 1996).

The current study also did not detect differences in chromosomal damage. Several other studies of BD-induced cytogenetic changes have produced inconsistent results. For example, the lack of positive findings from the current study both agree and disagree with the results of other

studies conducted at the same BD monomer production facility: Sorsa and coworkers (1994) found no changes in SCEs in their total study population, but found higher levels of chromosomal aberrations in a subset of BD-exposed workers lacking the *GSTT1* gene. In the same population, Šrám and colleagues (1998) reported increases in chromosomal aberrations and SCEs (including HFCs) in exposed compared with unexposed workers. BD exposures did not differ in these two studies. (The findings of those two studies are described in the earlier section Scientific Background / Genetic Polymorphisms and Cytogenetic Damage.) Possible reasons for the differing results are discussed in the Investigators' Report section Biomarkers of Effect: Cytogenetic Analysis. In a study of BD-exposed Chinese workers, Hayes and coworkers (2000) also found no cytogenetic damage and concluded that their study did not show BD-induced genotoxic effects. However, they cautioned that their negative results are relevant only for exposures in the range of BD studied (median level for exposed workers was 4.4 mg/m^3 [2 ppm]) and that the relatively small size of their study population limited their ability to detect modest effects.

In their concluding Discussion, Albertini and colleagues acknowledge the conflicting results of this and other studies on BD-induced changes in levels of biomarkers of effect. Nevertheless, they conclude that "the current study adds to the weight of evidence against genotoxicity for the population as a whole at the low exposure levels encountered in the modern BD industry. It does not offer new evidence for upgrading the classification of BD to a known human carcinogen (Class 1; IARC 1999) nor does it support the EPA's recent classification of BD as a known human carcinogen by inhalation (EPA 2002)." However, the number of subjects available and exposures that were lower than initially expected may have limited the statistical power for some mutational endpoints.

On the basis of their results of *HPRT* VFs in another study (Ammenheuser et al 2001), Ward and colleagues concluded that BD exposures between 2.2 mg/m^3 and 6.6 mg/m^3 (1 to 3 ppm) may be associated with adverse biological effects (discussed in the Scientific Background above). The mean TWA BD exposure for the polymer group in the current study was 1.76 mg/m^3 (0.8 ppm), which approaches Ward and coworkers' lower limit of 2.2 mg/m^3 (1 ppm). The individual TWAs of BD exposure for polymerization workers reached as high as 39.03 mg/m^3 (17.66 ppm). Thus, a study with greater statistical power might have detected elevated levels of *HPRT* mutations, should they have occurred.

SUMMARY

The main objective of this transitional epidemiologic study was to determine whether the appearance of a series of biomarkers in the blood and urine of workers exposed occupationally to BD could reliably indicate their level of exposure. If successful, such biomarkers could be used in full-scale epidemiologic studies.

The investigators studied the appearance of biomarkers of exposure and effects, and analyzed some other factors that may affect susceptibility in workers in the Czech Republic exposed occupationally in a BD monomer production facility and an adjacent SBR polymerization facility. Administrative workers in the company served as control subjects.

This challenging and comprehensive transitional epidemiologic study had several strengths and advantages.

- An experienced group of core investigators conducted the study.
- The design benefited from a pilot study that determined the steps needed to provide investigators in other countries with blood and urine samples prepared in the Czech Republic.
- The extensive exposure assessment was critically important in establishing the foundation upon which the biomarker measurements were interpreted.
- The investigators selected multiple endpoints for both biomarkers of exposure and biomarkers of effect.
- Multiple biomarkers of exposure (urinary metabolites and Hb adducts) were used to reflect different durations of exposure and were integrated with high-quality exposure data collected on the same individuals over the time span appropriate to the biomarkers.
- State-of-the-art analytic methods were used to quantify biomarker levels.

As with any study, there were accompanying limitations (some of which were not under the investigators' control).

- The number of subjects and exposure levels that were lower than initially expected limited the statistical power of certain analyses.
- The administrative control subjects apparently were not completely unexposed because they did experience measurable BD exposures and showed biomarkers of exposure in their blood and urine.
- Pharmacokinetic modeling of BD metabolism would have led to improved understanding and enhanced the usefulness of the data collected.

This important study linked BD exposure, measured by conventional sampling techniques, with biological markers

of that exposure. The investigators identified a set of objectives that were clear and achievable. Their study design was well crafted to effectively achieve their objectives and, in general, their conclusions are well supported by the data. The Investigators' Report contains a wealth of information that will be of great interest and use to researchers. The key findings of the study follow.

- Hb adducts (biomarkers of exposure) were highly correlated with BD exposure levels measured by passive samplers and integrated over time. THBVal adducts (from BDO-diol or BDO_2) were 300-fold to 400-fold higher than HBVal adducts (from BDO). Nevertheless, both may prove to be useful markers for exposure measurements in the workplace.
- Urinary metabolites (biomarkers of exposure) were also correlated with BD exposure levels integrated over time. M1 levels were 50-fold to 100-fold higher than M2 levels, as would be expected given that the human metabolic pathway for BD preferentially results in M1. Although these correlations were not as strong as those for Hb adducts, urinary metabolites may also prove to be useful markers of BD exposure.
- Some specific genotypes analyzed for loci that could affect susceptibility to carcinogens were associated with quantitative differences in dose-response relations of urinary metabolites or Hb adducts, although none indicated a heightened susceptibility to BD exposure.
- Biomarkers of effect (such as *HPRT* MFs, *HPRT* VFs, or other endpoints of chromosomal changes) showed no statistically significant increases or correlations with BD exposure levels.

The finding that both types of biomarkers of exposure reflected BD exposure levels in this study agrees with the results of Hayes and coworkers (2000). Those investigators also reported that (1) BD-exposed workers in China had higher levels of THBVal adducts than control workers and the adduct levels tended to correlate with BD exposure levels, and (2) urinary M1 levels were significantly correlated with BD exposure levels.

This study did not detect differences in mutation levels or chromosomal changes, but it was not designed to detect small differences. On the basis of these findings, the authors concluded that "the current study adds to the weight of evidence against genotoxicity for the population as a whole at the low exposure levels encountered in the modern BD industry. It does not offer new evidence for upgrading the classification of BD to a known human carcinogen (Class 1; IARC 1999) nor does it support the EPA's recent classification of BD as a known human carcinogen by inhalation (EPA 2002)." As discussed earlier, the small number of subjects and the exposure levels that were lower than expected may have limited the statistical power for some mutational and cytogenetic endpoints. Furthermore, none of the biomarkers was evaluated as a possible indicator or predictor of health outcomes. Thus, the genotoxicity of BD exposure at low levels remains uncertain.

This study by Albertini and colleagues was a very important and valuable transitional epidemiologic study because it established the linkage between exposure to BD, measured by comprehensive conventional sampling techniques, and several biological markers of that exposure. The integration of a comprehensive exposure assessment with a series of logical biomarker analyses was an outstanding feature of this complex, international study. Of the many biomarkers analyzed, biomarkers of exposure (particularly Hb adducts) will likely be valuable in future epidemiologic studies of the health effects of BD.

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