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An Epidemiologic Study of Early Biologic Effects of Benzene in Chinese Workers

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Benzene is a recognized hematotoxin and leukemogen, but its mechanisms of action in humans are still uncertain. To provide insight into these processes, we carried out a cross-sectional study of 44 healthy workers currently exposed to benzene (median 8-hr time-weighted average; 31 ppm), and unexposed controls in Shanghai, China. Here we provide an overview of the study results on peripheral blood cell levels and somatic cell mutation frequency measured by the glycophorin A (GPA) gene loss assay and report on peripheral cytokine levels. All peripheral blood cell levels (i.e., total white blood cells, absolute lymphocyte count, platelets, red blood cells, and hemoglobin) were decreased among exposed workers compared to controls, with the exception of the red blood cell mean corpuscular volume, which was higher among exposed subjects. In contrast, peripheral cytokine levels (interleukin-3, interleukin-6, erythropoietin, granulocyte colony-stimulating factor, tissue necrosis factor-α) in a subset of the most highly exposed workers ($n = 11$) were similar to values in controls ($n = 11$), suggesting that benzene does not affect these growth factor levels in peripheral blood. The GPA assay measures stem cell or precursor erythroid cell mutations expressed in peripheral red blood cells of MN heterozygous subjects, identifying NN variants, which result from loss of the GPA M allele and duplication of the N allele, and Nφ variants, which arise from gene inactivation. The NN (but not Nφ) GPA variant cell frequency was elevated in the exposed workers compared with controls (mean ± SD, 13.9 ± 8.4 mutants per million cells versus 7.4 ± 5.2 per million cells, respectively; $p = 0.0002$), suggesting that benzene produces gene-duplicating but not gene-inactivating mutations at the GPA locus in bone marrow cells of exposed humans. These findings, combined with ongoing analyses of benzene macromolecular adducts and chromosomal aberrations, will provide an opportunity to comprehensively evaluate a wide range of early biologic effects associated with benzene exposure in humans. — Environ Health Perspect 104(Suppl 6):1365–1370 (1996)

Key words: benzene, hydroquinone, muconic acid, hematotoxicity, cytokines, biomarkers, molecular epidemiology, somatic cell mutations, leukemogenesis

Introduction

Occupational exposure to benzene has been associated with hematotoxicity, leukemia, and other related blood disorders (*1–5*). Studies in animals and cell culture have produced a large amount of information on the potential mechanism of benzene toxicity, but the extrapolation of these data to humans requires further study. To provide insight into benzene's biologic effects in humans, we performed a cross-sectional study of 44 healthy workers exposed to a wide range of benzene levels and 44 unexposed controls in Shanghai, China, as part of a large research effort to evaluate benzene-related effects in a retrospective cohort study of 74,828 workers exposed to benzene in 12 Chinese cities (*4,5*). Some of the questions being addressed by this study are as follows:

a) What is the nature of benzene metabolites found at different levels of exposure, i.e., are proportionally more toxic benzene metabolites formed at higher or lower exposure levels?

b) What is the impact of interindividual variation in cytochrome P4502E1 (CYP2E1) activity on the formation of benzene metabolites and subsequent effects?

c) What is the pattern of hematotoxic effects?

d) Since certain cytokines are involved in regulating hematopoiesis, does benzene exposure alter cytokine levels in peripheral blood?

e) Does benzene form DNA adducts in peripheral white blood cells?

f) Does benzene nonspecifically cause chromosome aberrations in peripheral lymphocytes, or are there selective categories of aberrations formed or only particular chromosomes affected?

g) Does benzene induce gene mutations in human bone marrow, and, if so, what is the nature of these mutations?

Table 1 describes the data we have collected on this study population, which include measures of historical and current benzene air levels, and biomarkers of exposure, biologically effective dose, early

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(continued)

Table 1. Evaluation of biologic effects in workers exposed to benzene in Shanghai, China, 1992.

Benzene-related event	Measurement
External benzene exposure	
Historical	Questionnaire, factory records
Current	Passive dosimeters
Benzene metabolites	*trans-trans*-Muconic acid, phenol, phenylmercapturic acid, catechol, hydroquinone, trihydroxybenzene
Benzene adducts	Albumin, hemoglobin, DNA
Inherited susceptibility	*CYP2E1, GSTM1, GSTT1, NQ01* polymorphisms
Acquired susceptibility	Red blood cell folate Tobacco use (questionnaire, urine cotinine) Alcohol use (questionnaire)
Hematotoxicity	Peripheral blood cells, plasma cytokine levels
Oxidative damage	Urine 8-hydroxyl-deoxyguanosine
Chromosome aberrations	Fluorescence *in situ* hybridization, G-banding, micronuclei
Somatic cell mutations	Glycophorin A, polymerase chain reaction-based assays
Hepatotoxicity	Liver function enzymes
Neurotoxicity	Questionnaire

biologic effects, and susceptibility. Here we provide an overview of the study and report the impact of benzene exposure on peripheral blood cell levels, peripheral cytokine levels, and mutations measured by the glycophorin A (GPA) gene loss mutation assay.

Methods

Subject Enrollment

Details of the study population have been reported previously (*6*). Workplaces were identified from public health district records and from exposure and demographic data previously collected for the cohort study (*4,5*). Three exposed factories were selected so that the study population would have a wide range of exposure to benzene [1 to ≥25 ppm as an 8-hr time-weighted average (TWA), similar to that found among workers in the cohort study]. One factory used benzene to solubilize natural rubber for subsequent production of rubber padding for printing presses, one factory used benzene to manufacture adhesive tape, and one factory applied benzene-based paint and varnish to wooden toys and boxes. Two workplaces in the same geographic area that did not use benzene or other chemicals associated with bone marrow toxicity were selected as control factories: one manufactured sewing machines and the other was an administrative facility. Controls were frequency-matched on age (5-year intervals) and gender to workers currently exposed to benzene. Exclusion criteria for all subjects included prior history of cancer, therapeutic radiation, chemotherapy, or current pregnancy. Ninety-four percent of eligible subjects selected for study were enrolled.

Exposure Assessment

Exposure was estimated using a modification of the method used for the cohort study (*7*). This estimate incorporated work histories obtained by interview and review of workplace employment records, benzene area measurements obtained from factory records, and information on annual amount of benzene used, percent benzene used in workplace solvents, and changes in manufacturing, ventilation, and personal protective equipment use. Cumulative exposure to benzene was calculated from the historical time-specific exposure estimates and the duration worked. Exposure was assessed blinded with respect to all outcome measures.

Current exposure to benzene was monitored by organic vapor passive dosimetry badges (3M, St. Paul, MN). These badges were worn by each worker in an exposed factory for a full workshift on 5 separate days during 1 to 2 weeks before phlebotomy. The majority of subjects in the factory that manufactured sewing machines were monitored for one shift; subjects in the administrative facility were not monitored. Badges were analyzed by gas chromatography with flame ionization detection for benzene, toluene, and xylene. Forty-three of 44 benzene-exposed workers provided a spot urine sample at the end of a work shift or at the end of the high-exposure period during their work shift.

Interview and Biologic Sample Collection

Study subjects were evaluated at the Shanghai Hygiene and Anti-Epidemic Center. They were asked to refrain from eating solid foods after dinner the night before and the morning of the clinical phase of the study. On the day of study, the protocol was explained to all potential participants, and informed consent was obtained using Institutional Review Board-approved procedures. Height and weight of each subject were measured.

Subjects provided a baseline urine sample and then received 250 mg chlorzoxazone to evaluate CYP2E1 function (*8,9*) (results to be reported elsewhere). Subjects drank only noncaffeinated soft drinks for the first 2.5 hr, after which they ate a light lunch. Complete urine collections were carried out over the next 8 hr. A 27-ml volume of blood was obtained, and several peripheral smears were made for each subject. Portions of each blood sample were inoculated as whole-blood cultures, harvested, and processed into slides for analysis of interphase and metaphase chromosome aberrations and micronuclei. A separate portion was stabilized with ascorbic acid for eventual folate analysis. A third portion was fractionated into plasma, white blood cells, and red blood cells.

Each subject was administered a questionnaire by a trained interviewer from the staff of the Shanghai Hygiene and Anti-Epidemic Center's Department of Occupational Health. Data collected included age, gender, current and lifelong tobacco use, alcohol consumption, medical history, occupational work history, and the number of days in the previous week that subjects experienced symptoms associated with solvent neurotoxicity.

Laboratory Analysis

Benzene urinary metabolites were measured using a modification of a previously reported isotope dilution gas chromatography–mass spectroscopy assay (*10*). ^{13}C-labeled analogues of *trans-trans*-muconic acid (muconic acid) and phenol, catechol and hydroquinone as sulfate and glucuronide conjugates were used as internal standards.

Peripheral blood samples were analyzed by a Coulter T540 blood counter. Five hematologic measurements are presented here; the total white blood cell count (WBC) ($\times 10^3$/μl blood), the absolute lymphocyte count ($\times 10^3$/μl blood), hemoglobin value (g/dl), the red blood cell count

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Abbreviations used: BMI, body mass index; CYP2E1, cytochrome P4502E1; ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony-stimulating factor; GPA, glycophorin A; IL, interleukin; MCV, mean corpuscular volume; RBC, red blood cell; TNF-α, tumor necrosis factor-α; TWA, time-weighted average; V_f, variant cell frequency; WBC, white blood cell.

(RBC) ($\times 10^6$/μl blood), and the platelet count ($\times 10^3$/μl blood). The mean corpuscular volume (MCV; μm^3) was calculated as the hematocrit/(RBC × 10).

Cytokine measurements were prioritized on the basis of alterations previously detected in patients with the myelodysplastic syndrome (*11–13*). Plasma levels of granulocyte colony stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor both tend to be elevated in patients with aplastic anemia (*13*). Therefore, because of the limited volume of plasma available, we chose to measure G-CSF alone. Plasma levels of cytokines were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Interleukin (IL)-3, G-CSF, IL-6, and tumor necrosis factor-α (TNF-α) ELISA kits were purchased from R & D Systems (Minneapolis, MN) and the erythropoietin ELISA kit was obtained from Genzyme (Cambridge, MA). The ELISA plates were read and cytokine concentrations calculated from the standard curves using a Dynatech MR 5000 plate reader.

Analysis of peripheral RBCs for the glycophorin A gene loss assay has been described (*14*). In brief, spherical, formalin-fixed erythrocytes were prepared in duplicate from MN individuals and from MM and NN controls as described (*15*). The BR6 assay of Langlois et al. (*15*) was used with slight modification for enumerating Nϕ and NN variants. Data are reported as the NN or Nϕ variant cell frequency (V_f), defined as the number of mutant cells per million counted.

Statistical Analysis

Summary data are reported as mean (SD) or as median (range). Group differences for benzene metabolites, peripheral blood counts, and continuous cytokine values were tested by the Wilcoxon rank-sum test, and group differences for categorical cytokine outcomes were tested by Fisher's exact test. Spearman rank order correlation was used to test associations between benzene exposure and each outcome

The GPA NN and Nϕ V_f data were normalized with a logarithmic transformation, and group differences were tested by Student's *t*-test. A dose–response relationship between GPA V_f values and cumulative benzene exposure was tested by multiple linear regression, with subjects within each exposure category (0, 1–100, 101–500, and >500 ppm-years) assigned the median exposure for that category. Age, gender, current and lifetime tobacco use, alcohol consumption, diagnostic X-ray exposure, and body mass index (BMI), a measure of obesity calculated as weight/height^2, were tested for potential confounding. Two-tailed *p*-values <0.05 were considered statistically significant.

Results

Demographics and Exposure Assessment

Demographic characteristics of the 44 workers currently exposed to benzene and controls were similar, including age, gender, current smoking and alcohol consumption, and BMI (Table 2). The mean (SD) of occupational exposure to benzene among the exposed subjects was 6.3 (4.4) years. These subjects were currently exposed to a wide range of benzene levels (median: 31 ppm as an 8-hr TWA, range: 1–328 ppm). The mean muconic acid urine level was 33.0 (28.0) μg/mg creatinine in the exposed workers ($n = 43$) and 0.3 (0.2) μg/mg creatinine in a subsample of controls tested ($n = 17$, $p < 0.0001$). Phenol, hydroquinone, and catechol urine levels were similarly elevated in exposed workers compared to controls (data not shown). Further, as we have previously reported (*6*), the proportion of benzene metabolites excreted as muconic acid and hydroquinone decreased from a median of 32% in workers exposed to <31 ppm benzene to 24% in workers exposed to >31 ppm ($p = 0.006$).

Hematotoxicity

Workers currently exposed to benzene had lower total WBC, absolute lymphocyte, platelet, and RBC counts than controls (Table 3). Among all peripheral blood counts, the most significant difference between exposed subjects and controls was observed for the absolute lymphocyte count. Compared to controls, hemoglobin values of exposed workers were nonsignificantly decreased, and the MCV was significantly elevated (Table 3). Among the exposed workers, total urine benzene metabolite levels were significantly correlated only with the absolute lymphocyte count ($r = -0.32$, $p = 0.04$) and the MCV ($r = 0.37$, $p = 0.01$). In contrast, there were no significant correlations between cumulative benzene exposure and peripheral blood cell counts (data not shown).

Table 2. Demographics of workers currently exposed to benzene and controls, Shanghai, China, 1992.[a]

	Exposed workers	Controls, unexposed
Total subjects, *n* (%)	44 (100)	44 (100)
Male	23 (52)	23 (52)
Female	21 (48)	21 (48)
Current cigarette smokers, *n* (%)		
Yes	21 (48)	21 (48)
No	23 (52)	23 (52)
Current alcohol drinkers, *n* (%)		
Yes	15 (34)	17 (39)
No	29 (66)	27 (61)
Age, mean years (SD)	35.3 (7.8)	35.4 (7.3)
BMI[b], mean (SD)	21.6 (3.2)	21.5 (2.3)

[a]Adapted from Rothman et al. (*6*). [b]Body mass index (weight/height^2).

Peripheral Cytokine Levels

A subsample of the most highly exposed quartile of currently exposed workers ($n = 11$, 8-hr TWA >55.0 ppm) and controls ($n = 11$) was selected to determine if peripheral cytokine values were associated with benzene exposure. Levels of IL-6, erythropoietin, and TNF-α were similar in exposed subjects and controls (Table 4). Levels of G-CSF and IL-3 were nondetectable in almost all subjects. Neither benzene urinary metabolites nor cumulative benzene exposure was associated with any cytokine level (data not shown).

GPA Somatic Cell Mutation Frequency

The mean age of the 24 MN subjects in the exposed group was 34.2 (6.8) years compared to 35.1 (6.7) years among the 23 MN controls. Gender distribution, smoking

Table 3. Hematologic values of workers currently exposed to benzene and controls, Shanghai, China, 1992.[a]

	Mean, SD	
	Exposed workers ($n = 44$)	Controls, unexposed ($n = 44$)
White blood cells, $\times 10^3$/μl	6.0 (1.9)	6.8 (1.7)**
Absolute lymphocyte count, $\times 10^3$/μl	1.5 (0.3)	1.9 (0.4)***
Platelets, $\times 10^3$/μl	127 (44)	166 (59)**
Hemoglobin, g/dl	13.8 (2.0)	14.4 (2.0)
Red blood cells, $\times 10^6$/μl	4.4 (0.6)	4.7 (0.6)*
Mean corpuscular volume, μm^3	91.4 (4.0)	88.9 (4.9)*

[a]Adapted from Rothman et al. (*6*). *$p < 0.05$ **$p < 0.01$; ***$p < 0.001$

Table 4. Peripheral cytokine levels in workers currently exposed to benzene and controls, Shanghai, China, 1992.

Cytokine	Exposed workers[a] (n=11)	Controls, unexposed (n=11)
Mean (SD) levels		
IL-6, pg/ml	1.5 (1.2)	1.7 (1.7)
Erythropoietin, mU/ml	11.6 (5.8)	11.1 (10.2)
TNF-α, pg/ml	2.3 (1.0)	2.6 (1.0)
Subjects with detectable values/total subjects		
IL-3	0/11	2/11
G-CSF	0/11	1/11

[a]The most heavily exposed quartile of exposed workers (8 hr TWA > 55 ppm) was selected for study. There were no significant differences between the two groups.

Table 5. Glycophorin A mutant frequencies in MN heterozygous subjects by lifetime cumulative benzene exposure category, Shanghai, China, 1992.[a]

Lifetime cumulative occupational benzene exposure, ppm-years	Study subjects, n	Mean (SD) NN V_f	Mean (SD) Nϕ V_f
0	23	7.4 (5.2)	8.8 (8.7)
1–100	7	11.7 (8.5)	7.0 (3.4)
101–500	8	14.1 (11.0)	9.8 (3.2)
>500	9	15.3 (6.1)	10.2 (5.3)
Test for trend[a]		p=0.005	p=0.31

[a]Adapted from Rothman et al. (*14*). [b]Test for trend by linear regression on logarithmically transformed data, adjusted for age and sex.

habits, and BMI were similar in both groups. As we have previously reported (*14*), the NN V_f was about twice as high among the benzene-exposed workers compared to controls (13.9 ± 8.4 mutants per million cells versus 7.4 ± 5.2 mutants per million cells, p=0.0002). In contrast, there was not a significant difference between the two groups for the Nϕ V_f (9.1± 4.2 mutants versus 8.8 ± 8.7 mutants per million, p=0.21). There was a highly significant association between lifetime cumulative benzene exposure and NN V_f (p=0.005, Table 5), which was minimally changed after adjustment for potential confounders. In contrast, the Nϕ V_f was not associated with cumulative benzene exposure (p=0.31, Table 5). Total benzene urinary metabolites were not significantly correlated with either the NN Vf (r=0.02, p=0.94) or Nϕ V_f (r=0.35, p=0.10).

Discussion

We conducted this study to evaluate early biologic effects associated with benzene exposure. Forty-four subjects currently exposed to a wide range of benzene and 44 controls were studied in 1992 in Shanghai, China. Past patterns of exposure to benzene were estimated and current patterns of exposure were measured for all subjects, demographic data were collected, and blood samples were obtained and extensively processed in a manner appropriate for the analysis of a broad range of biomarkers.

Urinary levels of benzene metabolite were substantially higher among the exposed population than among controls. In addition, a larger proportion of benzene urinary metabolites were present as muconic acid and hydroquinone at lower (≤31 ppm) versus higher (≥31 ppm) levels of exposure, similar to reports in animal studies (*16*). This is particularly noteworthy because muconic acid and hydroquinone are correlated with benzene metabolites that have particularly potent genotoxic and myelotoxic effects (*17–22*).

The WBC, absolute lymphocyte, and platelet counts were significantly lower in benzene-exposed workers compared to controls; of these, the absolute lymphocyte count was the most significantly reduced in the exposed workers (27% lower compared to controls, p<0.001). Hemoglobin values were nonsignificantly depressed, and the MCV was significantly elevated. These findings are consistent with most previous reports of benzene hematotoxicity [reviewed by Goldstein (*23,24*) and Aksoy (*3*)]. An early report of Goldwater (*25*) demonstrated that the absolute lymphocyte count is particularly sensitive to benzene exposure, and studies have shown similar findings in animals exposed to as little as 10 ppm benzene (*26*). Total benzene urinary metabolites were significantly correlated with the absolute lymphocyte count and the MCV, while cumulative benzene exposure was not associated with any peripheral blood cell, suggesting that benzene-associated hematotoxicity reflects only relatively recent exposure.

The regulation of hematopoiesis is a dynamic process in which stem and progenitor cells, in conjunction with bone marrow stroma, give rise to a large number of mature blood cells. The survival and proliferation of stem and progenitor cells are controlled by multiple growth factors, or cytokines, with overlapping functions that act individually or in combination to regulate hematopoiesis. A series of studies carried out in mice and in human bone marrow cultures have shown that benzene alters cytokine production or response to cytokines in the bone marrow (*27–35*). To determine if benzene exposure affects cytokine levels measured in peripheral blood, a more accessible and acceptable tissue source than bone marrow, we evaluated a panel of cytokines in the most heavily exposed workers and a subset of controls. Cytokine levels were similar in the two groups.

Previous studies have shown that G-CSF, IL-6, erythropoietin, and TNF-α are elevated in patients with myelodysplastic syndromes (*11–13*). It is unclear if these markers have contributed to the disease state itself or reflect a feedback loop in response to altered blood counts. Our data suggest, within the limitations of the small sample size, that benzene exposure did not affect peripheral blood cytokine values, even if alterations in these growth factors occurred in the bone marrow. Further, if peripheral cytokine levels reflect only a hemostatic response to altered blood cell levels, the mild hematotoxicity present in the exposed workers may not have been severe enough to elicit this effect.

We measured GPA mutation frequency in peripheral RBCs from 24 workers exposed to benzene and in 23 unexposed controls. Since mature red blood cells lack a nucleus, mutations expressed in red blood cells must have occurred exclusively in precursor erythroid cells or stem cells in the bone marrow. The frequency of NN but not Nϕ mutants increased progressively with rising lifetime cumulative benzene exposure, suggesting that benzene causes repeated damage to longer-lived stem cells in human bone marrow. Since these stem cells are considered pluripotent, our finding has potential relevance for understanding the mechanism of DNA damage responsible for benzene-associated leukemia. NN variants could arise from mitotic recombination, gene conversion, or chromosome loss with reduplication. The most likely cause of gene-duplicating mutations measured in somatic cell mutation assays is mitotic recombination (*36*), which recent studies suggest plays an

important role in the development of leukemias and lymphomas (*37,38*).

We continue to analyze data collected from this study population to gain additional insight into the biologic effects of benzene exposure in humans (Table 1). For example, we report in the current volume that benzene exposure was associated with hyperdiploidy of chromosome 9 measured by fluorescent *in situ* hybridization in interphase lymphocytes (*39*), which was inversely correlated with the absolute lymphocyte count. This provides evidence of a link between hematotoxicity and aneuploidy in exposed humans and is consistent with previous reports that benzene poisoning (i.e., hematotoxicity) is associated with a high risk of subsequently developing leukemia (*40,41*).

In addition, we are currently analyzing similar data collected from a group of 50 subjects with a history of benzene poisoning (most of whom were no longer exposed to benzene) and controls. This work will provide an opportunity to examine the persistence of benzene-associated hematotoxicity, somatic cell mutations, and chromosome aberrations, and to evaluate susceptibility factors for these events.

Finally, we note that individual benzene air levels in these factories were much higher than expected based on historical area monitoring data. As a result of data collected for this study, remedial action was taken at the workplaces with the highest benzene exposures, which included substitution of toluene for benzene, improvement in ventilation, and enclosure of reaction vessels.

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