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Benzene induces gene-duplicating but not gene-inactivating mutations at the glycophorin A locus in exposed humans

(somatic cell mutations/biomarker)

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ABSTRACT **Occupational exposure to benzene is known to cause leukemia, but the mechanism remains unclear. Unlike most other carcinogens, benzene and its metabolites are weakly or nonmutagenic in most simple gene mutation assays. Benzene and its metabolites do, however, produce chromosomal damage in a variety of systems. Here, we have used the glycophorin A (GPA) gene loss mutation assay to evaluate the nature of DNA damage produced by benzene in 24 workers heavily exposed to benzene and 23 matched control individuals in Shanghai, China. The GPA assay identifies stem cell or precursor erythroid cell mutations expressed in peripheral erythrocytes of MN-heterozygous subjects, distinguishing the NN and NØ mutant variants. A significant increase in the NN GPA variant cell frequency (V_f) was found in benzene-exposed workers as compared with unexposed control individuals (mean ± SEM, 13.9 ± 1.7 per million cells vs. 7.4 ± 1.1 per million cells in control individuals; P = 0.0002). In contrast, no significant difference existed between the two groups for the NØ V_f (9.1 ± 0.9 vs. 8.8 ± 1.8 per million cells; P = 0.21). Further, lifetime cumulative occupational exposure to benzene was associated with the NN V_f (P = 0.005) but not with the NØ V_f (P = 0.31), suggesting that NN mutations occur in longer-lived bone marrow stem cells. NN variants result from loss of the GPA M allele and duplication of the N allele, presumably through recombination mechanisms, whereas NØ variants arise from gene inactivation, presumably due to point mutations and deletions. Thus, these results suggest that benzene produces gene-duplicating mutations but does not produce gene-inactivating mutations at the GPA locus in bone marrow cells of humans exposed to high benzene levels. This finding is consistent with data on the genetic toxicology of benzene and its metabolites and adds further weight to the hypothesis that chromosome damage and mitotic recombination are important in benzene-induced leukemia.**

Occupational exposure to benzene has been associated with aplastic anemia, leukemia, and other related blood disorders (1–3). Despite extensive research, the method by which benzene produces this toxicity remains unclear. Unlike most other carcinogens, benzene and its phenolic metabolites are weakly or nonmutagenic in most simple gene mutation assays (1, 4–7). Benzene and its metabolites do, however, produce chromosomal damage in a variety of systems. For example, they induce chromosomal aberrations, micronuclei, and sister chromatid exchanges in human lymphocytes *in vitro* (8–15). Many studies have also shown that benzene produces both structural and numerical chromosomal aberrations in the peripheral blood lymphocytes of occupationally exposed individuals (refs. 16–18, among others). Because consistent chromosomal aberrations have been observed in human leukemias and lymphomas, the induction of chromosomal damage by benzene or its metabolites may be critical in the development of benzene-induced leukemia. However, the nature of mutations produced at specific gene sites in the bone marrow, the target organ for benzene toxicity, has not been studied to date in humans.

Here, we have used the glycophorin A (GPA) mutation assay to detect different types of mutagenic events in the bone marrow of workers occupationally exposed to benzene. The GPA assay measures somatic cell mutation frequency in peripheral erythrocytes and is one of several phenotype somatic cell mutation assays available to study human populations (19–21). Because mature erythrocytes lack a nucleus, mutations expressed in erythrocytes must have occurred exclusively in precursor erythroid cells or stem cells in the bone marrow; this is particularly relevant for studying the effects of benzene because bone marrow cells are the target site for benzene-induced leukemia. The GPA assay is thought to detect a wide spectrum of mutational mechanisms, including point mutations, deletions, and chromosome-wide events or autosomal chromosome interactions, such as mitotic recombination (20). These mechanisms are especially important in the loss of heterozygosity of tumor-suppressor genes, as reviewed by Grant *et al.* (22).

The GPA locus is located on chromosome 4 and codes for an erythrocyte lineage-specific surface protein, which exists in two allelic forms, M and N (23). The GPA assay measures the frequency of variant cells that have lost expression of the M form in blood samples from heterozygous (MN) individuals (23). Variant cells are detected by treating sphered, fixed erythrocytes with fluorescent-labeled monoclonal antibodies specific for the M and N forms and counting variant cells that bind the anti-N antibody but do not bind the anti-M antibody by flow cytometry (20, 23). The variant cells possess the phenotype NØ, single-copy expression of N and no expression of M, or NN, double-copy expression of N and no expression of M. These phenotypic variants arise from different mutational mechanisms in precursor cells: NØ cells are thought to arise from point mutations, deletions, or gene inactivation, whereas NN cells presumably arise from mitotic recombination, chromosome loss and reduplication, or gene conversion (20, 23). Thus, the ability of benzene to produce different types of mutations in the bone marrow can be tested with this assay.

We have evaluated GPA mutation frequencies in 24 workers exposed to benzene and 23 control individuals in Shanghai,

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Abbreviations: GPA, glycophorin A; V_f, variant cell frequency; BMI, body mass index.

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China. Here we report that benzene exposure increases NN variant cell frequency (V_f) but does not increase NØ V_f and conclude that benzene causes recombination-type mutations in bone marrow stem cells in humans.

MATERIALS AND METHODS

Subject Enrollment. The study was approved by the Institutional Review Boards of the Chinese Academy of Preventive Medicine, the U.S. National Cancer Institute, and the University of California, Berkeley. Forty-four workers currently exposed to benzene from three workplaces, with minimal exposure to other aromatic solvents, were enrolled in the study in 1992. Controls were selected from two workplaces in the same general area, which had negligible benzene exposure. Exclusion criteria included history of cancer, therapeutic radiation, or chemotherapy. The 44 controls were frequency-matched on age (5-yr intervals) and sex to the benzene-exposed group. Because no females and almost all males were current smokers, the benzene-exposed subjects and controls were similar with regard to smoking.

The study was supervised by U.S. and Chinese investigators on-site. The protocol was explained to all potential participants, and informed consent was obtained. Each subject was administered a questionnaire by trained interviewers from the staff of the Department of Occupational Health, Shanghai Hygiene and Anti-Epidemic Center. Data collected included age, sex, current and lifelong tobacco use, alcohol consumption, medical history, history of diagnostic x-ray exposure, and work history. Height and weight of each subject were measured, a 27-ml sample of blood was obtained by venous phlebotomy, and spot urine samples were obtained.

Industrial Hygiene Assessment. The workers were enrolled from three factories in Shanghai that process natural rubber padding for printing presses, manufacture adhesive tape, and paint and varnish wooden toys and boxes, respectively. Individual current benzene exposure was monitored by organic vapor passive dosimetry badges (3M no. 3500, St. Paul) worn by each worker for a full work shift on 5 separate days during a 1- to 2-week period before phlebotomy. Although some workers wore half-face respirators, these were not considered effective because fit testing was not done, and workers frequently smelled benzene inside their masks. Badges were analyzed for benzene by gas chromatography with flame ionization detection, and 8-hr time-weighted-average benzene exposure was calculated by determining the geometric mean of the five air measurements. No other chemical or physical carcinogens were identified in these workplaces.

Peripheral blood from each subject was analyzed by a Coulter Counter (model T540) for absolute lymphocyte count. Study subjects in the benzene-exposed workplaces provided a spot urine sample at the end of their work shift or at the end of the high-exposure period during their work shift. The benzene metabolite phenol, which is a good indicator of absorbed benzene at the levels in these factories, was measured in urine samples by using a modification of a previously described isotope dilution gas chromatography/mass spectroscopy (GC/MS) assay (24). ^{13}C-labeled analogues of phenol as sulfate and glucuronide conjugates were used as internal standards. Concentrations were determined by calibrating against a primary standard solution. The analytes were derivatized with bistrimethylsilytrifluoracetamide and analyzed by GC/MS.

Historical benzene exposure during subjects' employment at the study factories was estimated by trained field personnel with methods similar to those developed to assess past benzene exposure in a large cohort study of ≈75,000 workers exposed to benzene (25). 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 ventilation practices. Historical estimates considered current individual exposure levels when historical exposure monitoring data were sparse. Discrete time intervals in each workplace were created based upon changes in manufacturing or ventilation practices. The geometric mean of air-level measurements obtained during a given time interval was multiplied times the number of years a subject worked during that time interval; exposure from all time periods a worker spent in a factory was then summed to generate a cumulative benzene exposure variable in ppm-yr. All exposure assessment was done blinded with respect to hematologic measurements.

GPA Assay. Within 0.5 hr of phlebotomy the MN blood type was determined by using rabbit typing serum (Ortho Diagnostics). MN heterozygous blood was kept for 1–2 hr at 4°C until formalin fixation. Spherical, formalin-fixed erythrocytes were prepared in duplicate from MN individuals and from MM and NN control individuals, according to the method of Langlois *et al.* (23). The fixed specimens were stored at 4°C until analysis. Analyses were done within 13.1 ± 3.5 days of collection on coded samples that were blinded with respect to exposure status.

Anti-N (BRIC 157) and anti-M (6A7) antibodies were obtained as cell-culture supernatant from BioProducts Laboratory Commercial Department (Elstree, U.K.) and were purified by ammonium sulfate precipitation and protein G (Pharmacia/LKB) chromatography, respectively. Fluorescent and biotin labelings were as described (23). The BR6 assay of Langlois *et al.* (23) was used with slight modification for enumerating NØ and NN variants. Sphered erythrocytes were incubated with anti-M (biotinylated 6A7) and anti-N (fluoresceinated BRIC 157) antibodies, washed, then incubated with avidin–phycoerythrin (Caltag, South San Francisco, CA), washed, and suspended in buffer for flow cytometry. MM and MØ variants cannot be measured with this assay because the anti-M antibody, 6A7, reacts with another cell-surface antigen, glycophorin B, giving rise to an MM and MØ cell population of stained erythrocytes too close to the large MN region in the fluorescence dot plot for accurate enumeration of the rare variants. Use of the Consort 32 software (Becton Dickinson) allows five million cells to be logged to a single data file and provides 256 channels along each of the FL1 (x) and FL2 (y) axes of the dot plots. Spherical, singlet erythrocytes were selected for analysis using a polygonal gate based on forward-scatter versus side-scatter dot plots. The NØ and NN windows were 24 channels wide out of 256 channels on the FL1 axis and were individually adjusted using the MN mean peak for each specimen. The windows on the FL2 axis were from the baseline to 40–77, depending on the result on the day's calibration with homozygous samples (23); the variation was due to different detector gain settings. All samples were analyzed in duplicate. The duplicates were independently fixed, stained, and analyzed on different days. Two analyses were discarded based on visual inspection of the raw data; these individual's variant frequencies are consequently based on a single determination. All others were averaged.

Statistical Methods. Summary values are presented as mean ± SD unless otherwise noted. Simple correlation analyses were done with Spearman rank–order correlation. The distribution of original, untransformed NN and NØ V_f data within the benzene-exposed and control groups is portrayed by using Box and Whisker plots. The NN and NØ V_f data were normalized with a logarithmic transformation, and group differences were tested by Student's t test. Analysis of covariance was used to test for V_f differences between groups after adjustment for demographic variables including age, sex, current and lifetime tobacco use, alcohol consumption, diagnostic x-ray exposure, and body mass index (BMI), a measure of obesity calculated as $\text{weight}_{kg}/\text{height}^2_{meter}$. Cumulative benzene exposure was transformed by the square root to place it in the general range of the other independent variables (e.g., age).

Table 1. Demographic characteristics of MN-heterozygous study subjects by exposure status

Demographic characteristic	Subjects, no. (%)	
	Benzene-exposed	Control
Total subjects	24 (100)	23 (100)
Age		
18–30	7 (29)	4 (17)
31–40	15 (63)	15 (65)
41–50	2 (8)	4 (17)
Sex		
Male	13 (54)	10 (43)
Female	11 (46)	13 (57)
Smoking status		
Never	12 (50)	15 (65)
Former	0 (0)	0 (0)
Current	12 (50)	8 (35)

A dose–response relationship between GPA V_f values and cumulative benzene exposure was tested by multiple linear regression, with subjects within each category of exposure (0, 1–100, 101–500, and >500 ppm-yr) assigned the median exposure for that category. Exposure categories were created by dividing the exposed subjects into approximate tertiles within even exposure cut-offs and was done before analyzing the GPA V_f data. Evaluation of a dose–response relationship by exposure category allowed us to determine whether there was a monotonic increase in GPA V_f from one exposure level to the next. Age and sex, the original matching variables, were retained in all models. The demographic variables described in the preceding paragraph were evaluated for their potential confounding of the dose–response relationship. Two-tailed P values <0.05 were considered statistically significant.

RESULTS

Twenty-four (55%) of the benzene-exposed group and 23 (52%) of the control group were heterozygous (MN) for the GPA locus and could therefore be analyzed for GPA mutation frequency. The mean ± SD age of the MN subjects in the exposed group was 34.2 ± 6.8 yr compared to 35.1 ± 6.7 yr among the MN controls. Sex distribution and smoking habits were similar (Table 1). Mean pack yr (average number of cigarette packs smoked per day × number of years of smoking) of cigarette use was 7.4 ± 5.3 pack yr among exposed smoking workers and 9.8 ± 7.2 pack yr among smoking controls. Self-reported current tobacco use positively correlated with urine cotinine levels (adjusted for creatinine) measured by GC/MS ($r = 0.85, P = 0.0001$). BMI was similar in both groups (22.4 ± 3.6 kg/m^2, exposed workers; 22.0 ± 2.1 kg/m^2, control individuals).

Table 2. Benzene-exposure characteristics among MN-heterozygous benzene-exposed workers

Exposure characteristic	Mean (SD)	Median (range)
Current 8-hr TWA benzene air levels, ppm	72.2 (65.1)	66.2 (2.4–301.1)
Urine phenol levels, μg/mg of creatinine	226.8 (175.3)	268.1 (30.3–516.7)
Cumulative occupational benzene exposure, ppm-yr	607.4 (779.6)	270.5 (8–3488)

TWA, time-weighted average.

Among the 24 MN benzene-exposed workers, mean duration of employment was 6.9 ± 4.9 yr (range, 0.7–16.5 yr). Table 2 describes current and historical benzene exposure in this population. Current benzene exposure was exceptionally high (median, 66 ppm) and positively correlated with urine phenol levels ($r = 0.63, P = 0.003$, for 20 workers who provided a urine sample on the same day air was monitored). Median lifetime cumulative benzene exposure was 270 ppm-yr (Table 2). Current and lifetime cumulative benzene exposure correlated with each other at $r = 0.49$ ($P = 0.01$).

The combined NN and NØ V_f was substantially higher among the benzene-exposed workers than among the control individuals (mean ± SEM; 23.0 ± 2.1 vs. 16.3 ± 2.3 per million cells, $P = 0.005$). This relationship was primarily due to differences in the NN V_f, which was about twice as high among the benzene-exposed workers compared with controls (mean ± SEM, 13.9 ± 1.7 vs. 7.4 ± 1.1 per million cells, $P = 0.0002$) (Fig. 1*A*). In contrast, there was not a significant difference between the two groups for the NØ V_f (mean ± SEM: 9.1 ± 0.9 vs. 8.8 ± 1.8 per million, $P = 0.21$) (Fig. 1*B*). These findings were unchanged after adjustment for age, sex, current, and lifetime tobacco use, BMI, alcohol consumption, and diagnostic x-ray exposure. There was a positive correlation between the NØ V_f and NN V_f, which was stronger but not statistically

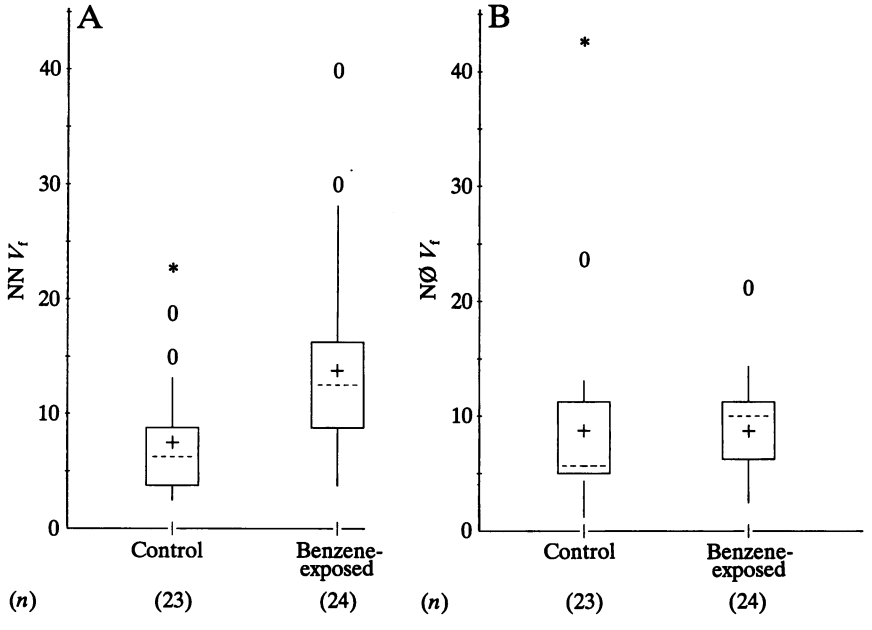

FIG. 1. (*A*) Comparison of NN V_f (per million cells) between workers exposed to benzene and controls. Upper edge of box represents highest 25th percentile of data; lower edge of box represents 75th percentile. Upper and lower lines indicate range of remaining data except for outliers. 0, Outlier defined as >1.5 and <3.0 times the distance between the 25th and 75th percentiles; *, extreme outlier defined as >3.0 times the distance between the 25th and 75th percentiles. +, Mean of all samples. ---, Median [difference by Student's t test ($P = 0.0002$) on logarithmic-transformed data]. (*B*) Comparison of NØ V_f (per million cells) between workers exposed to benzene and controls [difference by Student's t test ($P = 0.21$) on logarithmic-transformed data].

significant among the exposed subjects ($r = 0.36$, $P = 0.09$ and $r = 0.19$, $P = 0.39$, for exposed and control subjects, respectively).

Because the windows for scoring NN and NØ variants are contiguous, slight variation in placing these windows can alter the designation of mutants at the NN/NØ interface. To determine whether the findings described above might be influenced due to difficulty in clearly separating each mutant class, we eliminated one channel on either side of the NN/NØ interface, resulting in a 7% decrease in the NN V_f and a 9% loss in the NØ V_f. Recalculated differences between exposed and unexposed subjects were unchanged (NN V_f difference, $P = 0.0001$; NØ V_f difference, $P = 0.23$). Subsequent analyses were done for NN and NØ V_f values separately because the preceding results suggested that benzene exposure affects these two mutation rates in different ways.

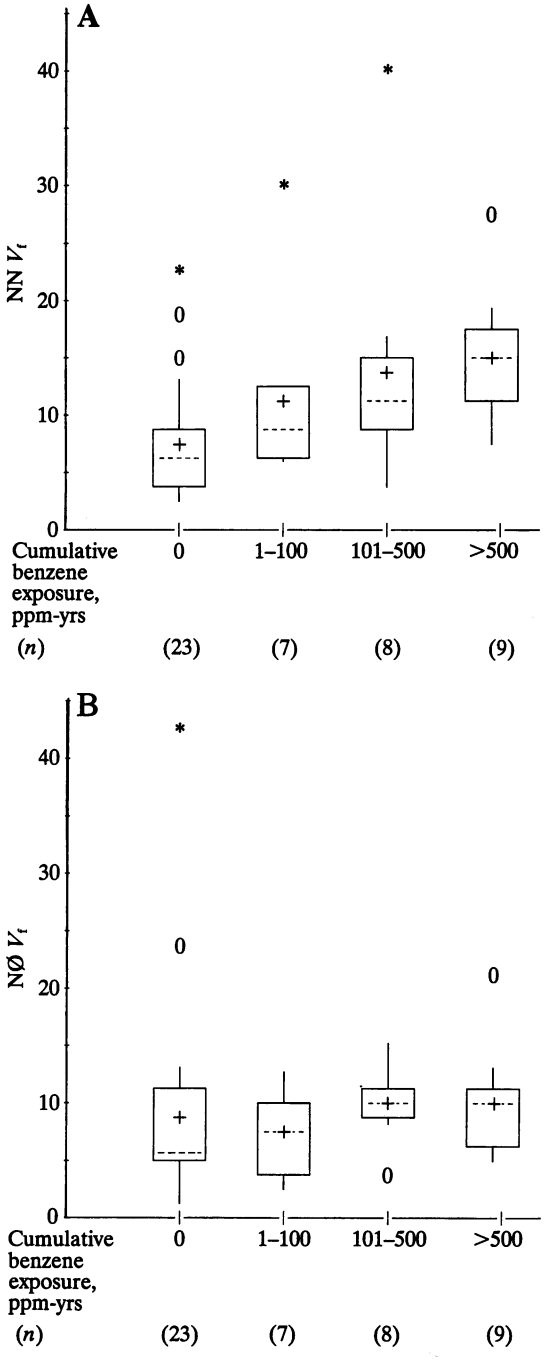

FIG. 2. (*A*) Correlation of NN V_f (per million cells) with cumulative lifetime benzene exposure. Test for trend ($P = 0.005$) by linear regression on logarithmic-transformed data, adjusted for age and sex. (*B*) Correlation of NØ V_f (per million cells) with cumulative lifetime benzene exposure. Test for trend ($P = 0.31$) by linear regression on logarithmic-transformed data, adjusted for age and sex. Symbols are as for Fig. 1.

The association between lifetime cumulative benzene exposure and NN V_f values categorized into 0, 1–100, 101–500, and >500 ppm-yr of benzene exposure is presented in Fig. 2*A*, which portrays a highly significant positive trend ($P = 0.005$). This was minimally changed after adjustment for the variables described above. Further, the difference in NN V_f values between the lowest (0 ppm-yr) and highest (>500 ppm-yr) category was highly significant ($P = 0.001$). Similar results were obtained by using noncategorized exposure data. The equation $\ln(\text{NN } V_f) = 0.024\ (\text{cumulative exposure})^{1/2} + 1.92$ was significant at $P = 0.0002$ ($P = 0.0008$, adjusted for age and sex). In contrast, there was no association detected between the NØ V_f and cumulative exposure ($P = 0.31$) (Fig. 2*B*) and no difference was seen between the lowest and highest exposure category ($P = 0.29$). With noncategorized exposure data, the equation $\ln(\text{NØ } V_f) = 0.0087\ (\text{cumulative exposure})^{1/2} + 1.89$ was not significant ($P = 0.21$), and this association was further weakened after adjustment for age and sex ($P = 0.37$).

Among the 24 benzene-exposed workers, the NN V_f positively correlated with lifetime cumulative benzene exposure as a continuous variable but did not correlate with current benzene exposure (Table 3). The opposite effects were seen with the peripheral absolute lymphocyte count, which was inversely correlated with current benzene air levels and urine phenol but was not correlated with cumulative exposure. In contrast, the NØ V_f value was not significantly correlated with any measure of benzene exposure (Table 3).

DISCUSSION

We measured GPA mutation frequency in peripheral erythrocytes from 24 workers exposed to benzene and in 23 unexposed controls. Overall, the GPA mutation frequency was higher in the benzene-exposed workers due to an increase in the frequency of NN variants. Furthermore, the frequency of NN but not of NØ mutants increased progressively with rising lifetime cumulative benzene exposure. Of note is that among the exposed workers, the NN mutation frequency correlated with cumulative but not with current benzene exposure, suggesting that benzene causes repeated damage to longer-lived stem cells in human bone marrow. Because bone marrow stem cells are considered pluripotent, our finding has potential relevance for understanding the mechanism of DNA damage responsible for benzene-associated leukemia. The absolute lymphocyte count inversely correlated with current but not with cumulative exposure, which was expected because peripheral white blood cell counts decline within several months of initial contact with high levels of benzene and generally return to baseline after benzene levels are reduced below hematotoxic levels (26, 27).

Table 3. Correlation of GPA mutant frequencies and peripheral lymphocyte count with measures of workplace benzene exposure ($n = 24$ workers)

	R (*P*)		
Exposure characteristic	NN V_f	NØ V_f	Lymphocyte count
Cumulative occupational benzene exposure, ppm-yr	0.42 (0.04)	0.21 (0.32)	−0.02 (0.92)
Current benzene 8-hr TWA air exposure, ppm	0.08 (0.70)	0.17 (0.42)	−0.36 (0.08)
Urine phenol levels, μg/mg of creatinine	−0.08 (0.71)	0.32 (0.13)	−0.44 (0.03)

TWA, time-weighted average.

NN variants could arise from mitotic recombination, gene conversion, or chromosome loss with reduplication. Thus, from these data, benzene appears to induce this class of mutation in the human bone marrow but does not produce point mutations, deletions, or other types of gene-inactivating mutations associated with NØ variants at the GPA locus. The most likely form of gene-duplicating mutation induced by benzene is mitotic recombination. Turner and coworkers (28) reported that most gene-duplicating spontaneous mutations in a similar human somatic mutation assay at the HLA-A locus were caused by mitotic recombination between the HLA locus and the centromere. To date, neither benzene nor its metabolites have been tested for their ability to induce mitotic recombination, but their DNA strand-breaking and clastogenic properties would suggest that they are recombinogenic.

Recent studies suggest that recombination plays an important role in the development of leukemias and lymphomas. Errors of DNA recombination in developing blood cells cause many of the chromosome translocations that characterize lymphoid tumors (29) and some leukemias (30). Because these translocations appear to be centrally involved in the development of the leukemic phenotype, further investigation of the ability of benzene and its metabolites to induce recombination is warranted.

Elevated frequencies at the GPA locus have been found in subjects exposed to ionizing radiation (31–33), in patients receiving anti-neoplastic drugs (34–36), and in people with cancer-prone syndromes (37–39). An excess of both NØ and NN variants was found in patients with Bloom syndrome (with NN variants higher than NØ) (37) and Fanconi anemia (39). An increase in only NØ variants was found among Chernobyl clean-up workers exposed to ionizing radiation (33); a greater increase was detected in NØ than in NN variants in germ-cell tumor patients receiving platinum-based chemotherapy (35); and, an increase was found for both variants among childhood Hodgkin disease patients receiving combination chemotherapy (36). In contrast, our study found a significant increase only for NN variants among benzene-exposed workers. These studies support the use of the GPA assay to gain insight into both the presence and nature of genetic damage in bone marrow cells caused by different carcinogenic agents. Further, we suggest that a similar mechanism may be involved in the production of genetic mutations in Bloom syndrome and by benzene because both result in increased levels of NN variants.

In conclusion, heavy benzene exposure produces a selective increase in NN mutations in the GPA assay. The most likely form of mutation produced is mitotic recombination in longer-lived stem cells. To the extent that benzene produces similar types of mutations in genes involved in leukemogenesis, such genetic alterations may be important in benzene-induced leukemia. Further studies are needed of larger populations of exposed workers to reproduce this finding and to examine the ability of benzene metabolites to induce mitotic recombination in human cells.

Finally, we would note that current exposure levels to benzene in these factories were much higher than we had expected to find at the outset of this study. These levels are not representative of exposure patterns in Chinese industry today, which decreased to an average of 8 ppm (8-hr time-weighted average) as of 1987 (25). Remedial action has been taken at two of the workplaces with the highest exposures and has included substitution of toluene for benzene, enclosure of reaction vessels, and improvement in ventilation.

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