

Increased Translocations and Aneusomy in Chromosomes 8 and 21 Among Workers Exposed to Benzene¹

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ABSTRACT

Chromosome aberrations in peripheral blood lymphocytes have been used for many years to monitor human populations exposed to potential carcinogens. Recent reports have confirmed the validity of this approach by demonstrating that elevated levels of chromosome aberrations in lymphocytes are associated with subsequent increased cancer risk, especially for increased mortality from hematological malignancies including acute myeloid leukemia (AML). We postulated that this approach could be improved in two ways: (a) by detecting oncogenic disease-specific aberrations; and (b) by using chromosome painting so that many more metaphases could be analyzed. Numerical and structural aberrations in chromosomes 8 and 21 are commonly observed in AML. In the present study, we painted chromosomes 8 and 21 in lymphocyte metaphases from 43 healthy workers exposed to benzene, an established cause of AML, and from 44 matched controls. To examine dose-response relationships the workers were divided into two groups at the median exposure level, a lower-exposed group (≤ 31 ppm; $n = 21$), and a higher-exposed group (> 31 ppm; $n = 22$). Benzene exposure was associated with significant increases in hyperdiploidy of chromosomes 8 (1.2, 1.5, and 2.4 per 100 metaphases; $P < 0.0001$) and 21 (0.9, 1.1, and 1.9 per 100 metaphases; $P < 0.0001$). Translocations between chromosomes 8 and 21 were increased up to 15-fold in highly exposed workers (0.01, 0.04, and 0.16 per 100 metaphases; $P < 0.0001$). In one highly exposed individual, these translocations were reciprocal and were detectable by reverse transcriptase-PCR. These data indicate a potential role for t(8;21) in benzene-induced leukemogenesis and are consistent with the hypothesis that detection of specific chromosome aberrations may be a powerful approach to identify populations at increased risk of leukemia.

INTRODUCTION

The measurement of chromosome aberrations in peripheral blood lymphocytes has been used for many years to monitor human populations exposed to carcinogens or potential carcinogens (1, 2). For example, there are numerous studies in the literature that show that human exposure to the leukemogen benzene increases the level of chromosome aberrations in lymphocytes detected by conventional methods (3–5). Recent reports have confirmed the validity of this approach by demonstrating that elevated levels of chromosome aberrations in peripheral blood lymphocytes are associated with subsequent increased cancer risk (6, 7), especially for increased mortality from hematological malignancies including AML³ (7).

Exposure to benzene in the workplace has been associated with increased risk of AML, aplastic anemia, and myelodysplastic syn-

dromes (8–10). A variety of specific chromosome aberrations, including trisomy of chromosomes 8 and 21 and translocations between these two chromosomes, are commonly found in AML and myelodysplastic syndromes (11–13). Translocation (8;21)(q22;q22) is the most common translocation observed in AML. It occurs in 40% of patients with the AML-M2 subtype and disrupts both the *AML1* (*CBFA2*) gene on chromosome 21 and the *ETO* (*MTG8*) gene on chromosome 8 (13, 14). The fusion transcript *AML1-ETO* has been isolated (15) and can be detected by RNA-based RT-PCR (16). Chromosome translocations can also be detected by chromosome painting using FISH (17). FISH has a number of advantages over PCR including the fact that not only structural but also numerical chromosomal aberrations can be visualized and precisely quantitated. On the other hand, PCR holds the potential advantage of greater sensitivity. FISH and RT-PCR, therefore, complement one another.

We propose that specific chromosomal aberrations that are known to be oncogenic may provide a better marker of future leukemia risk than conventional chromosomal aberrations. As an initial test of this hypothesis, we have used FISH and RT-PCR to determine the presence of AML-specific chromosomal aberrations in the peripheral blood of workers who are at increased risk of AML because they are exposed to high levels of benzene and in the peripheral blood of matched controls. We demonstrate that AML-specific aberrations are found at increased levels in the blood cells of workers exposed to benzene and propose that the detection of disease-specific chromosomal aberrations by chromosome painting and RT-PCR may be a powerful approach to identifying populations at increased risk of leukemia.

MATERIALS AND METHODS

Subject Enrollment and Exposure Assessment. The enrollment of subjects and identification of factories for this study in Shanghai, China has been previously described in detail by Rothman *et al.* (18, 19). Forty-four exposed workers and 44 matched controls were enrolled in this study. Informed consent was obtained from each subject, and a questionnaire was administered to determine age, gender, current and lifelong tobacco use, current alcohol consumption, medical history, and work history. Exclusion criteria for all subjects were history of cancer, therapeutic radiation, chemotherapy, or current pregnancy. Three factories that used benzene were selected so that the study population would have a wide range of exposures to benzene. 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 factory manufactured sewing machines, whereas the second workplace was an administrative facility.

Individual exposure was monitored by passive dosimetry badges, which were worn by each worker for a full workshift on 5 separate days during the 1–2-week period before phlebotomy. Badges were analyzed by gas chromatography with flame ionization detection. An 8-h TWA exposure to benzene was calculated as the geometric mean of the five air measurements. The median TWA was 31 ppm (range, 1–328 ppm). Current exposures to benzene were confirmed by the analysis of phenol and other metabolites in the urine (19). These exposures were reflective of workplace exposures for at least 1 year before the study, as determined from a review of factory work practice records (20). Cumulative exposure to benzene while working in these factories

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³The abbreviations used are: AML, acute myeloid leukemia; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcriptase-PCR; TWA, time-weighted average.

employed was also assessed as described previously (19, 21). All workers had been employed for at least 8 months, and their job practices were extremely stable. Remedial action was taken subsequently at the two workplaces with the highest benzene exposures. This included the substitution of toluene for benzene, the enclosure of reaction vessels, and an improvement in ventilation.

Blood Sample Collection and Culture. Peripheral blood was obtained by phlebotomy; cultured in RPMI 1640 supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine (Life Technologies, Inc., Grand Island, NY), and 1% phytohemagglutinin-P (Pharmacia, Piscataway, NJ) at 37°C in a 5% CO₂ moist atmosphere; and harvested 72 h after culture initiation. Colcemid (0.1 µg/ml) was added 4 h before harvesting to obtain the metaphase spreads. After hypotonic treatment (0.075 M KCl) for 30 min at 37°C, the cells were fixed three times with fresh Carnoy's solution (methanol:glacial acetic acid, 3:1). The fixed cells were then dropped onto prelabeled glass slides, air dried, stored at -20°C under nitrogen, and shipped on dry ice to the United States. Blood from one person could not be successfully cultured, and results are reported here for only 87 subjects. Blood smears were also prepared and used for the PCR-based analyses.

FISH Analysis of Metaphase Spreads. Dual-color painting of whole chromosomes 8 (green) and 21 (red) was performed on metaphase spreads prepared from cultured lymphocytes. The biotinylated human painting probe specific for chromosome 8 and the digoxigenin-labeled painting probe for chromosome 21 were purchased from Oncor, Inc. (Gaithersburg, MD). After the denaturation of cellular DNA in 70% formamide in 2× SSC at 72°C for 2 min, the slides were quickly dipped in ice-cold 70, 85, and 100% ethanol for 2 min each to dehydrate them and then air dried. After prewarming for 5 min at 37°C, each of the painting probes was denatured separately for 10 min at 70°C and then preannealed for 2.5 h at 37°C. The differently labeled probes of chromosomes 8 and 21 were mixed well, applied to prewarmed slides (at 37°C) on a slide warmer, and hybridized overnight with target DNA at 37°C in a humidified chamber.

The slides were washed the next day in 0.5× SSC at 72°C for 5 min after three brief washes in 0.1 M phosphate buffer (pH 8.0) at room temperature. The hybridization signals were detected in a mixed solution of 10 µg/ml FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) in preblock solution (4× SSC, 0.1% Triton X-100, 0.02% sodium azide, and 5% Carnation nonfat dry milk) and 10 µg/ml antidigoxigenin antibody conjugated with rhodamine (Boehringer Mannheim, Indianapolis, IN) in phosphate buffer with 4% BSA for 15 min at 37°C. After three washes in phosphate buffer for 2 min each with intermittent agitation at room temperature, the nuclei of the cells were counterstained with a blue fluorescent dye, 4',6-diamidino-2-phenylindole (0.1 µg/ml; Sigma, St. Louis, MO), prepared in a mounting medium (Vector Laboratories). The hybridization signals were viewed under a fluorescence microscope equipped with epifluorescent illumination and a ×100 oil immersion lens. A triple-bandpass filter for 4',6-diamidino-2-phenylindole/FITC/Texas Red (excitation at 405, 490, and 570 nm; emission at 460, 525, and 635 nm) was used.

The stained slides were randomized and coded before scoring. For efficiency, all scorable metaphase spreads on two spots of each slide were analyzed. Metaphase cells were considered scorable if they met the following criteria: (a) the cells appeared to be intact; (b) the chromosomes were condensed and well spread; (c) the centromeres of all chromosomes were readily visible; and (d) staining was sufficiently bright to enable the detection of genetic rearrangements on chromosomes 8 and 21 and exchanges between targeted and nontargeted chromosomes. A total of 42,082 metaphase spreads from the 87 subjects (average, 483 cells/subject) were scored for the presence of numerical and structural aberrations in chromosomes 8 and 21. This is approximately 10 times more than the number routinely scored by conventional cytogenetics (50 cells/subject).

Molecular Analysis of Blood Smears for the Presence of AML1/ETO Chimeric cDNA Derived from t(8;21). One-half of the contents of coded blood smear slides previously stored at room temperature were scraped into microcentrifuge tubes, and total RNA was isolated with TRIzol reagent (Life Technologies, Inc.). Approximately 0.5–1.2 µg of RNA were isolated by this method, as determined by A_{260 nm}. One-third of the RNA was added to 50 ng of random primers, heated to 95°C for 5 min, quick-cooled on ice, and then added to a mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 50 mM DTT, 25 pmol each of four deoxynucleotide triphosphates, 20 units of RNasin, and 50 ng of random hexanucleotide. Moloney murine

leukemia virus reverse transcriptase (200 units; Promega, Madison, WI) was added to a total volume of 20 µl. The mixture was incubated at room temperature for 10 min and then incubated at 37°C for 60 min. Negative controls included samples with all components but the reverse transcriptase or RNA. The positive controls used were the Kasumi-1 cell line (22) kindly provided by Dr. Nanao Kamada (Research Institute of Nuclear Medicine and Biology, Hiroshima, Japan) and RNA isolated from two AML patients with t(8;21).

The cDNA mixture was PCR amplified with the primers AML1/ETO5A (GACCTCAGGTTTGTCTCGTCTGAAG) and AML1/ETO3A (CCATTGCTGAAGCCATTGGGTGG) using a hot start protocol. Five µl of cDNA, 25 pmol of each primer, 5% DMSO, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 pmol of each deoxynucleotide triphosphate, and 2.5 units of Taq polymerase (Perkin-Elmer Corp., Foster City, CA) were amplified for 39 cycles of PCR (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 72°C for 20 min). A second nested reaction using 1 µl of the first reaction was performed using the 5'B and 3'B primers described previously (16). The second PCR reaction was electrophoresed through 2% agarose, blotted to nylon membranes (Genescreen; DuPont), and probed by a ³²P-labeled oligonucleotide probe encompassing the junction of the fusion transcript. Quality of the RNA was assessed by the ability to PCR-amplify β-actin and hypoxanthine phosphoribosyltransferase genes from the cDNA. PCR products from the external reaction (5A and 3A primers) were directly sequenced using the 5'B and 3'B primers (PCR Product Sequencing Kit; Amersham). Both strands were sequenced, and the results were verified to the published deletion junction sequence (15).

Statistical Analysis. Study subjects were divided into three groups: (a) controls; (b) workers exposed to ≤31 ppm of benzene; and workers exposed to >31 ppm of benzene as an 8-h TWA. We have previously shown that these exposure categories are highly correlated with urinary benzene metabolite levels and various measures of hematotoxicity (18). Summary data for aneuploidy of chromosomes 8 and 21 within these exposure groups are presented as the mean of the number of aberrations per 100 metaphases scored for each subject. A square root transformation was used to normalize each outcome, and a test for trend was performed by linear regression, controlling for age and gender, the original matching variables. There was no evidence of confounding by current or previous tobacco use, alcohol use, or body mass index, and these factors were excluded from final models. Because structural chromosomal aberrations occurred at 1–2 orders of magnitude less frequently than aneuploidy, the data were pooled for all subjects within each exposure category. Summary data are presented as the total number of events detected over the total number of metaphases scored within each group, and a test for trend was performed using exact methods.

RESULTS

Demographics and Exposure Levels. Exposed workers and controls had similar demographic characteristics with mean ± SD ages of 35.3 ± 7.8 and 35.4 ± 7.3 years, respectively. Forty-eight percent of each group were women. None of the women in either group smoked, but 21 of 23 men in both the exposed and control groups were smokers. The exposed males smoked 11.0 ± 7.8 cigarettes/day, and nonexposed males smoked 13.5 ± 13.7 cigarettes/day. Both groups had a low mean alcohol intake of approximately 1.5 drinks/week. The workers exposed to benzene had worked in their respective factories for a mean of 6.3 ± 4.4 years, with a range of 0.7–16 years. The median current benzene air exposure level was 31 ppm. The exposed workers were divided into two groups at the median exposure level, a lower-exposed group (≤31 ppm) and a higher-exposed group (>31 ppm), to evaluate the dose-response relationships. Cumulative exposure was assessed in terms of ppm-years. Fifteen exposed subjects (34%) had 1–100 ppm-years of exposure, 16 (36%) had 100–500 ppm-years of exposure, and 13 (30%) had >500 ppm-years of exposure.

Aneusomy of Chromosomes 8 and 21. Numerical chromosome aberrations (aneuploidy/aneusomy) can be divided into two categories: (a) hyperdiploidy (a gain in the chromosome number); or (b)

hypodiploidy (a decrease in the chromosome number). Benzene exposure was associated with a highly significant increasing trend in hyperdiploidy of both chromosomes 8 and 21 in the lymphocytes of exposed workers ($P < 0.0001$; Table 1). In the higher-exposure category, the rise in hyperdiploidy was 2-fold. The effect of benzene on both chromosomes was approximately equal and was mainly in the form of trisomy. In contrast to the significant correlation observed between current benzene exposure and hyperdiploidy, no correlation was found with cumulative exposure.

As expected, hypodiploidy was considerably more prevalent than hyperdiploidy in the metaphase spreads. Exposure to benzene caused a small but significant increase in the hypodiploidy of chromosome 8 ($P = 0.0007$) but not that of chromosome 21 ($P = 0.84$; Table 1). Interestingly, the background level of chromosome 21 hypodiploidy was 2- to 3-fold higher than that of chromosome 8 (Table 1).

Structural Aberrations in Chromosomes 8 and 21. Three types of structural aberrations in chromosomes 8 and 21 could be detected by chromosome painting; namely, breaks, deletions, and translocations. Breaks were observed as painted acentric fragments. Unpainted acentric fragments were excluded from the analysis. Deletions were detected by comparing the length of two chromosomes painted in the same color. Three types of translocations could be detected by painting: (a) translocations between chromosomes 8 and 21 [t(8;21)]; (b) translocations between chromosome 8 and another unidentified chromosome [t(8;?)] and (c) translocations between chromosome 21 and another unidentified chromosome [t(21;?)]. The frequency of these different types of structural aberrations is shown in Fig. 1 using pooled metaphase data. Benzene caused a highly significant increase in chromosome 8 breaks ($P < 0.0001$), but not in deletions ($P = 0.48$; Fig. 1). The level of t(8;21) was increased 15-fold in workers exposed to >31 ppm over the level found in control subjects. It was also significantly higher in the lower-exposed group (≤ 31 ppm) than it was in the controls. The increase in other translocations, i.e., t(8;?) and t(21;?), was smaller at around 4-fold in the group exposed to > 31 ppm and was not significantly higher in the group exposed to ≤ 31 ppm as compared to controls (Fig. 1).

To further explore the distribution of aberrations within the different exposure categories shown in Fig. 1, the data are presented in Table 2 as the proportion of subjects in each exposure group with at least one detectable event. A tendency toward an increased number of individuals with detectable structural aberrations in their blood in the exposed groups is clearly observable (Table 2). Note that 2% of the control subjects (1 individual) had detectable t(8;21), whereas 19% of the workers in the ≤ 31 ppm lower-exposed group (four individuals) and 27% of workers in the higher (>31 ppm)-exposed group (six individuals) had detectable t(8;21). Similar increases in the number of individuals with t(8;?), t(21;?), and chromosome 8 breaks were also observed.

To determine whether individuals with the highest level of structural aberrations in their blood biased this analysis, we repeated the trend tests excluding the subject with the highest value in each category. Similar highly significant trends were found: t(8;21), $P = 0.00013$; t(8;?), $P < 0.0001$; t(21;?), $P = 0.045$; and chromosome

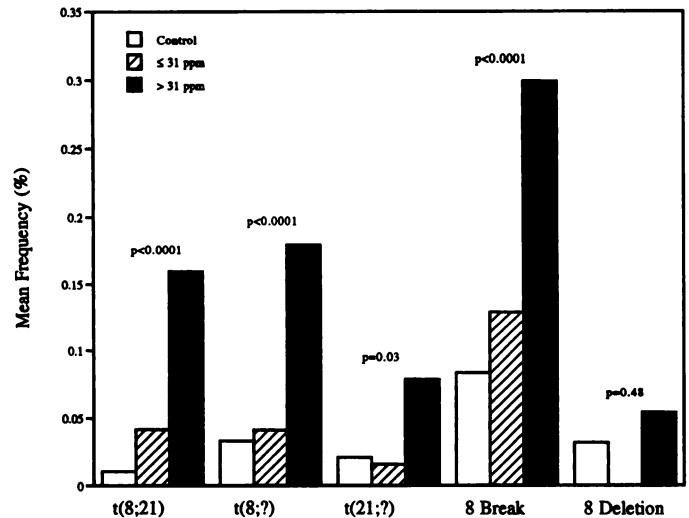


Fig. 1. Structural chromosome aberrations in chromosomes 8 and 21 in lymphocytes from the blood of workers exposed to different levels of benzene and unexposed controls, as detected by chromosome painting. P values equal to trend test by exact methods.

8 breaks, $P < 0.0001$. This demonstrates that outliers or subjects with clonal abnormalities are not the cause of the elevated levels of structural aberrations seen in the benzene-exposed workers as a whole. Interestingly, the worker with the highest level of structural chromosome aberrations did not have the highest level of t(8;21). However, the worker with the highest level of t(8;21) (7 of 100 metaphases) was also the only individual to have detectable reciprocal translocations between chromosomes 8 and 21 (2 of 100 metaphases). In all other t(8;21)-positive individuals (10 of 43 individuals), part of chromosome 21 was translocated to chromosome 8, or part of chromosome 8 was translocated to chromosome 21, but no reciprocal exchanges were detectable.

Molecular Analysis of t(8;21) by RT-PCR. To further characterize the t(8;21) translocations described above, we performed a molecular analysis using RT-PCR of RNA isolated from the blood of a subset ($n = 21$) of 11 highly exposed workers and 10 matched unexposed controls (Fig. 2). Five members of the exposed group and one member of the unexposed group had t(8;21) detectable by FISH. The groups were matched on age, gender, and smoking status. RNA was isolated from coded blood smear slides and reverse-transcribed, and *AML1/ETO* chimeric cDNA was PCR-amplified in a nested reaction. PCR products were blotted and probed with a junction-specific oligonucleotide and sequenced for confirmation. The experiment was performed blind, so that lanes on the gel were identified after the fact. In one exposed individual who had reciprocal t(8;21) detectable by FISH, we were able to detect the *AML1-ETO* fusion transcript, demonstrating that the t(8;21) translocation detected by FISH in this worker's blood was in-frame (Fig. 2). Sequencing showed that it was identical to that found in the t(8;21)-positive Kasumi-1 cell line and two AML patients positive for t(8;21) by G-banding analysis. We did not detect fusion transcripts in other

Table 1. Aneuploidy of chromosomes 8 and 21 in benzene-exposed workers and matched controls

Benzene exposure (n)	Chromosome 8		Chromosome 21	
	Hypodiploidy	Hyperdiploidy	Hypodiploidy	Hyperdiploidy
Controls (44)	5.8 ± 3.2 ^a	1.2 ± 0.8	14.2 ± 8.7	0.9 ± 0.7
≤31 ppm (21)	6.2 ± 2.7	1.5 ± 0.8	15.2 ± 6.6	1.1 ± 0.6
>31 ppm (22)	8.9 ± 4.8	2.4 ± 1.1	13.7 ± 5.4	1.9 ± 0.9
Trend (P value) ^b	0.0007	<0.0001	0.84	<0.0001

^a Mean ± SD events per 100 scored metaphase cells.

^b P values obtained by linear regression on square root-transformed values, adjusted for age and gender.

Table 2. Distribution of structural aberrations involving chromosomes 8 and 21 in benzene-exposed workers and matched controls

Benzene exposure (n)	t(8;21)	t(8;?) ^a	t(21;?) ^b	Breakage of chromosome 8	Deletion in chromosome 8
Controls (44)	1 ^c (2) ^d	5 (11)	4 (9)	10 (23)	5 (11)
≤31 ppm (21)	4 (19)	4 (19)	1 (5)	7 (33)	0 (0)
>31 ppm (22)	6 (27)	11 (50)	8 (36)	12 (55)	5 (23)

^a Translocation between chromosome 8 and another unidentified chromosome.^b Translocation between chromosome 21 and another unidentified chromosome.^c Number of subjects with specific structural change.^d Percentage of subjects with specific structural change.

exposed workers or control subjects (Fig. 2). RNA was amplified from all subjects using β -actin and hypoxanthine phosphoribosyl-transferase primers showing that failure to detect fusion transcripts was not due to a lack of RNA (data not shown).

DISCUSSION

Acute leukemia is often characterized by the presence of specific numerical and structural chromosome aberrations in the malignant clone. For example, malignant clones in AMLs commonly have numerical changes in chromosomes 5, 7, and 8 or contain translocations between chromosomes 8 and 21 (11–13, 23, 24). These chromosomal changes confer a selective advantage to proliferate, resist differentiation, and/or survive apoptosis, and the malignant clone of myeloid origin becomes the dominant WBC type in the blood and marrow (25, 26). It is widely believed that cells of the lymphocytic lineage do not possess the same clonal abnormalities as those found in the myeloid lineage (27), but they may be present at far lower levels, undetectable by conventional methods. Because we prepared metaphases from whole blood, it is possible that the specific chromosome aberrations we detected in the present study were in a small population of myeloid cells. This is highly unlikely, however, because myeloid cells are terminally differentiated and will not divide in response to phytohemagglutinin or survive 72 h of culture. Because AML-specific changes will not confer a selective growth advantage on lymphoid cells (28), the presence of AML-specific chromosomal changes in lymphocytes in nondiseased individuals reflects the rate at which these events are being produced by genotoxic insults. Furthermore, because lymphocytes are much longer-lived than granulocytes, the production of aberrations in the former is cumulative over a period of months. The quantitation of AML-specific aberrations in peripheral blood lymphocytes may therefore provide a measure of damage and leukemia risk incurred by exposure to genotoxic agents such as benzene and radiation and to the environment in general. This idea is a subtle modification of the long-established approach of monitoring

conventional chromosome aberrations in the peripheral blood lymphocytes of people exposed to potential carcinogens.

The use of chromosome aberrations as biomarkers of early effect recently received substantial support from the findings of Hagmar *et al.* (6) and Bonassi *et al.* (7) that showed that high levels of chromosome aberrations in peripheral blood lymphocytes were associated with an increased risk of subsequently developing cancer, especially hematological malignancies (7). We reasoned that this approach could be improved in two ways: (a) by detecting oncogenic disease-specific aberrations; and (b) by using FISH, so that many more metaphases could be analyzed, thereby increasing the statistical power. As an initial test of this hypothesis, we have used FISH to study metaphase spreads prepared from the lymphocytes of workers exposed to benzene. Chromosome painting by FISH allowed us to detect aberrations specific to AML, notably hyperdiploidy 8 and t(8;21). It also allowed us to study an average of 483 metaphases/individual, an order of magnitude more metaphases than is routinely scored by conventional cytogenetics. The results of this analysis showed that exposure to benzene was associated with increased levels of hyperdiploidy of chromosome 8, mainly in the form of trisomy 8, and translocations between chromosomes 8 and 21 in the lymphocytes of the exposed but otherwise healthy workers. Benzene exposure also increased the number of breaks in chromosome 8 and the number of translocations between chromosomes 8 and 21 and other chromosomes. Translocation (8;21) was increased as much as 15-fold in the highly exposed workers.

Benzene exposure was associated not only with increased levels of specific aberrations in the groups as a whole but also with a dose-dependent increase in the number of individuals with detectable structural aberrations. This is illustrated by the fact that there was a dramatic increase in the percentage of the population with detectable translocations in the high-dose group. For example, t(8;21) was observed in only 2% of control subjects but was detected in 27% of the high-dose group (Table 2). Likewise, the percentage of the population



Fig. 2. Southern blot to detect the presence of *AML1/ETO* chimeric cDNA derived from t(8;21) in the blood of workers exposed to high levels of benzene and unexposed controls. Amplification and blotting of cDNA prepared from RNA isolated from the 11 benzene-exposed workers is shown in Lanes 3, 5, 10, 11, 14–18, 21, and 24. Five of these workers had t(8;21) detectable by FISH (Lanes 3, 5, 10, 11, and 24). A positive response is seen in Lane 24, showing that this worker had detectable *AML1/ETO* fusion transcripts in his peripheral blood. RNA from the 10 matched unexposed controls was amplified and blotted in Lanes 1, 2, 4, 8, 12, 13, 19, 20, 22, and 23. One of these unexposed workers had t(8;21) detectable by FISH, but a positive response was not observed (Lane 12). RNA isolated from two t(8;21)-positive leukemia patients and the Kasumi-1 cell line [which harbors the t(8;21) translocation] was included in Lanes 6, 9, and 25, respectively, as positive controls. RNA from one t(8;21)-negative leukemia patient was included as a negative control (Lane 7). The analysis was performed blind, so that the lanes were identified after the fact.

with t(8;?) rose from 11 to 50% (Table 2). Thus, in the high-dose group, one-fourth to one-half of the population had detectable translocations by the FISH methodology described here, whereas a much smaller proportion (approximately one-tenth) had detectable translocations of any kind in the unexposed controls.

Interestingly, increased levels of specific chromosome aberrations were correlated only with current exposure, reflective of exposures over the previous 6–12 months, and not to cumulative exposure levels measured in ppm-years. This would tend to indicate that the majority of the aberrations detected had a half-life of a few months at most and thus would not be detectable many years after exposure. We are currently conducting a study using the same methodology of workers with past exposure, but no current exposure, to high levels of benzene to expand on this finding. However, the present results are quite different from those reported in this same population using the glycophorin A mutation assay, which measures mutations in erythroid progenitors in the bone marrow, in which cumulative exposure but not current exposure correlated with increased mutant frequency (21). The fact that different cell types are affected in the production of glycophorin A mutations and specific chromosome aberrations may explain this difference. The results are also inconsistent with the prevailing theory that all translocations are persistent stable aberrations. However, the recent findings of Spruill *et al.* (29) suggest that some translocation frequencies decline with time and are not completely persistent (30).

Chromosome painting has previously been used to study humans and cell lines exposed to ionizing radiation (31, 32). The effects of radiation are considered to be random, so that the number of breaks and translocations in a particular chromosome is proportional to the size of that chromosome. Indeed, it has been argued that the effects of radiation on the whole genome can be studied using only two or three chromosomes (31). If breaks and subsequent translocations were random, one would expect that translocations between chromosomes 8 and 21 would occur 60 times less frequently than translocations between chromosome 8 and all of the other chromosomes and 19 times less frequently than translocations between chromosome 21 and other chromosomes. These values were calculated using the formulas devised by Savage and Papworth (33) and Lucas *et al.* (34) using the genomic contents of human chromosomes described by Morton (35). In fact, a striking selectivity for stable translocations between chromosomes 8 and 21 was observed in controls as well as in exposed workers. In controls, the ratio of t(8;?):t(8;21) was only 3, not 60; and the ratio of t(21;?):t(8;21) was only 2, not 19 (Fig. 1). Thus, in unexposed controls, chromosome 8 was 10–20 times more likely to exchange with chromosome 21 than with other chromosomes. In the workers exposed to lower levels of benzene (≤ 31 ppm), the ratio of t(8;?):t(8;21) fell to 1 (0.042:0.042), and for t(21;?):t(8;21), it was even lower (0.4; 0.017:0.042). Similar ratios (1.125 and 0.5, respectively) were observed in the highly exposed workers (> 31 ppm). Thus, in the benzene-exposed workers, chromosome 8 was 38–60 times more likely to exchange with chromosome 21 than with other chromosomes, suggesting that some type of site-specific recombination or selection process must be occurring. However, the ratio of 38–60-fold in benzene-exposed workers was not significantly greater than the ratio of 10–20-fold found in controls.

The increased frequency of t(8;21) in the workers highly exposed to benzene detected by FISH encouraged us to attempt to detect *AML1-ETO* fusion transcripts in the blood of these workers by RT-PCR. *AML1* and *ETO* are the two genes that are fused in the translocation (8;21). Only limited material was available for this analysis in the form of 4-year-old blood smears. However, we have recently been able to detect gene expression by RT-PCR in stored Guthrie card blood spots; therefore, we applied this same technology (36, 37) to

study the blood smears. RNA was isolated from the blood smears of a subset of 21 exposed workers and matched controls. We were only able to detect identifiable *AML1-ETO* fusion transcripts in the RNA isolated from the blood smear of the worker with the highest level of t(8;21), and the only subject with reciprocal translocations detected by FISH. This fusion transcript was identical in sequence to that found in the Kasumi-1 cell line. This demonstrates that the translocation detected by FISH was in-frame and of etiological significance to leukemogenesis. RT-PCR therefore confirmed the FISH analysis, showing the power of this combined approach.

RT-PCR and PCR have previously been used to detect translocations (9;22) and (14;18) in unexposed individuals of different ages and in smokers (38, 39). Both translocations were found to increase with age, and the t(14;18) translocation was increased in cigarette smokers. We are not aware of any previous studies showing detectable t(8;21) by RT-PCR in nondiseased, untreated individuals. The results obtained here and those described previously by others clearly demonstrate the potential of RT-PCR in monitoring specific aberrations in populations exposed to suspected or established leukemogens. Future studies are planned to collect samples from workers exposed to benzene and from the general population to examine the level of different translocations in their peripheral blood by RT-PCR.

The mechanism by which benzene exposure leads to increased levels of translocations between chromosomes 8 and 21 is not clear at present. One possible mechanism may be related to topoisomerase II inhibition, because t(8;21) is a clonal abnormality associated with AML caused by topoisomerase-inhibiting drugs (28), and benzene metabolites are topoisomerase II poisons (40). We are currently performing further analysis of the metaphase spreads collected from these populations to measure changes at chromosome loci known to contain topoisomerase recognition sites, including 11q23, and at sites on the genome also related to leukemia development such as 5q31 and 7q22.

In summary, we have shown that benzene exposure is associated with markedly elevated levels of t(8;21) and of hyperdiploidy 8 and 21, in the circulating lymphocytes of otherwise healthy workers exposed to benzene compared to unexposed controls. This suggests a role for these aberrations in benzene-induced leukemia and that their detection in peripheral blood cells by chromosome painting may be useful biomarkers of increased risk for hematological malignancy for benzene and other potential leukemogens. Prospective studies are being considered to further test this hypothesis.

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REFERENCES

- Galloway, S. M., Berry, P. K., Nichols, W. W., Wolman, S. R., Soper, K. A., Stolley, P. D., and Archer, P. Chromosome aberrations in individuals occupationally exposed to ethylene oxide, and in a large control population. *Mutat. Res.*, 170: 55–74, 1986.
- Littlefield, L. G., and Joiner, E. E. Analysis of chromosome aberrations in lymphocytes of long-term heavy smokers. *Mutat. Res.*, 170: 145–150, 1986.
- Tough, I. M., and Court Brown, W. M. Chromosome aberrations and exposure to ambient benzene. *Lancet*, 7387: 684–685, 1965.
- Forni, A. Chromosome changes and benzene exposure. A review. *Rev. Environ. Health.*, 3: 5–17, 1979.
- Major, J., Jakab, M., Kiss, G., and Tompa, A. Chromosome aberration, sister-chromatid exchange, proliferative rate index, and serum thiocyanate concentration in smokers exposed to low-dose benzene. *Environ. Mol. Mutagen.*, 23: 137–142, 1994.

6. Hagmar, L., Brogger, A., Hansteen, I. L., Heim, S., Hogstedt, B., Knudsen, L., Lambert, B., Linnainmaa, K., Mitelman, F., Nordenson, I., Reuterwall, C., Salomaa, S., Skerfving, S., and Sorsa, M. Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic Study Group on the Health Risk of Chromosome Damage. *Cancer Res.*, 54: 2919-2922, 1994.
7. Bonassi, S., Abbondandolo, A., Camurri, L., Dal Pra, L., De Ferrari, M., Degrossi, F., Forni, A., Lamberti, L., Lando, C., Padovani, P., Sbrana, I., Vecchio, D., and Puntoni, R. Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study. *Cancer Genet. Cytogenet.*, 79: 133-135, 1995.
8. Aksoy, M. Benzene Carcinogenicity, pp. 113-151. Boca Raton, FL: CRC Press, Inc., 1988.
9. Hayes, R. B., Yin, S. N., Dosemeci, M., Li, G. L., Wacholder, S., Travis, L. B., Li, C. Y., Rothman, N., Hoover, R. N., and Linet, M. S. Benzene and the dose-related incidence of hematologic neoplasms in China. Chinese Academy of Preventive Medicine-National Cancer Institute Benzene Study Group. *J. Natl. Cancer Inst.*, 89: 1065-1071, 1997.
10. Rinsky, R. A., Smith, A. B., Hornung, R. F., Filloon, T. G., Young, R. J., Okun, A. H., and Landrigan, P. J. Benzene and leukemia. An epidemiologic risk assessment. *N. Engl. J. Med.*, 316: 1044-1050, 1987.
11. Pedersen-Bjergaard, J., and Rowley, J. D. The balanced and the unbalanced chromosome aberrations of acute myeloid leukemia may develop in different ways and may contribute differently to malignant transformation. *Blood*, 83: 2780-2786, 1994.
12. Sandberg, A. A. The Chromosomes in Human Cancer and Leukemia, 2nd ed., pp. 223-312. New York: Elsevier, 1990.
13. Kagan, J. Molecular biology of chromosomal aberrations in leukemia/lymphoma. *Hematol. Pathol.*, 7: 159-201, 1993.
14. Nucifora, G., and Rowley, J. D. AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. *Blood*, 86: 1-14, 1995.
15. Erickson, P., Gao, J., Chang, K. S., Look, T., Whisenant, E., Raimondi, S., Lasher, R., Trujillo, J., Rowley, J., and Drabkin, H. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to *Drosophila* segmentation gene, runt. *Blood*, 80: 1825-1831, 1992.
16. Downing, J. R., Head, D. R., Curcio-Brint, A. M., Hulshof, M. G., Motroni, T. A., Raimondi, S. C., Carroll, A. J., Drabkin, H. A., Willman, C., Theil, K. S., Civin, C. I., and Erickson, P. An AML1/ETO fusion transcript is consistently detected by RNA-based polymerase chain reaction in acute myelogenous leukemia containing the (8;21)(q22;q22) translocation. *Blood*, 81: 2860-2865, 1993.
17. Gray, J. W., and Pinkel, D. Molecular cytogenetics in human cancer diagnosis. *Cancer (Phila.)*, 69: 1536-1542, 1992.
18. Rothman, N., Li, G.-L., Dosemeci, M., Bechtold, W., Marti, G. E., Wang, Y.-Z., Linet, M., Xi, L.-Q., Lu, W., Smith, M. T., Titenko-Holland, N., Zhang, L., Blot, W., Yin, S., and Hayes, R. Hematotoxicity among Chinese workers heavily exposed to benzene. *Am. J. Ind. Med.*, 29: 236-246, 1996.
19. Rothman, N., Smith, M. T., Hayes, R. B., Li, G. L., Irons, R. D., Dosemeci, M., Haas, R., Stillman, W. S., Linet, M., Xi, L. Q., Bechtold, W. E., Wiemels, J., Campleman, S., Zhang, L., Quintana, P. J., Titenko-Holland, N., Wang, Y. Z., Lu, W., Kolachana, P., Meyer, K. B., and Yin, S. An epidemiologic study of early biologic effects of benzene in Chinese workers. *Environ. Health Perspect.*, 6 (Suppl. 104): 1365-1370, 1996.
20. Dosemeci, M., Li, G. L., Hayes, R. B., Yin, S. N., Linet, M., Chow, W. H., Wang, Y. Z., Jiang, Z. L., Dai, T. R., Zhang, W. U., Chao, X.-J., Ye, P.-Z., Kou, Q.-R., Fan, Y.-H., Zhang, X.-C., Lin, X.-F., Meng, J.-F., Zho, J.-S., Wacholder, S., Kneller, R., and Blot, W. J. Cohort study among workers exposed to benzene in China. II. Exposure assessment. *Am. J. Ind. Med.*, 26: 401-411, 1994.
21. Rothman, N., Haas, R., Hayes, R. B., Li, G. L., Wiemels, J., Campleman, S., Quintana, P. J., Xi, L. J., Dosemeci, M., Titenko-Holland, N., Meyer, K. B., Lu, W., Zhang, L., Bechtold, W., Wang, Y. Z., Kolachana, P., Yin, S. N., Blot, W. J., and Smith, M. T. Benzene induces gene-duplicating but not gene-inactivating mutations at the glycophorin A locus in exposed humans. *Proc. Natl. Acad. Sci. USA*, 92: 4069-4073, 1995.
22. Asou, H., Tashiro, S., Hamamoto, K., Otsuji, A., Kita, K., and Kamada, N. Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. *Blood*, 77: 2031-2036, 1991.
23. Le Beau, M. M., Espinosa, R. D., Neuman, W. L., Stock, W., Roulston, D., Larson, R. A., Keinanen, M., and Westbrook, C. A. Cytogenetic and molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases. *Proc. Natl. Acad. Sci. USA*, 90: 5484-5488, 1993.
24. Le Beau, M. M., Espinosa, R. D., Davis, E. M., Eisenbart, J. D., Larson, R. A., and Green, E. D. Cytogenetic and molecular delineation of a region of chromosome 7 commonly deleted in malignant myeloid diseases. *Blood*, 88: 1930-1935, 1996.
25. Russell, N. H. Biology of acute leukaemia. *Lancet*, 349: 118-122, 1997.
26. Irons, R. D., and Stillman, W. S. The process of leukemogenesis. *Environ. Health Perspect.*, 6 (Suppl. 104): 1239-1246, 1996.
27. Knuutila, S., Teerenhovi, L., Larramendy, M. L., Elonen, E., Franssila, K. O., Nylund, S. J., Timonen, T., Heinonen, K., Mahlamaki, E., Winqvist, R., and Ruutu, T. Cell lineage involvement of recurrent chromosomal abnormalities in hematologic neoplasms. *Genes Chromosomes Cancer*, 10: 95-102, 1994.
28. Pedersen-Bjergaard, J., Pedersen, M., Roulston, D., and Philip, P. Different genetic pathways in leukemogenesis for patients presenting with therapy-related myelodysplasia and therapy-related acute myeloid leukemia. *Blood*, 86: 3542-3552, 1995.
29. Spruill, M. D., Ramsey, M. J., Swiger, R. R., Nath, J., and Tucker, J. D. The persistence of aberrations in mice induced by γ radiation as measured by chromosome painting. *Mutat. Res.*, 356: 135-145, 1996.
30. Matsumoto, K., Ramsey, M. J., Nelson, D. O., and Tucker, J. D. Persistence of radiation-induced translocations in human peripheral blood determined by chromosome painting. *Radiat. Res.*, in press, 1998.
31. Lucas, J. N., Awa, A., Straume, T., Poggensee, M., Kodama, Y., Nakano, M., Ohtaki, K., Weier, H. U., Pinkel, D., Gray, J., and Littlefield, G. Rapid translocation frequency analysis in humans decades after exposure to ionizing radiation. *Int. J. Radiat. Biol.*, 62: 53-63, 1992.
32. Matsuoka, A., Tucker, J. D., Hayashi, M., Yamazaki, N., and Sofuni, T. Chromosome painting analysis of X-ray-induced aberrations in human lymphocytes *in vitro*. *Mutagenesis*, 9: 151-155, 1994.
33. Savage, J. R., and Papworth, D. G. Frequency and distribution studies of asymmetrical versus symmetrical chromosome aberrations. *Mutat. Res.*, 95: 7-18, 1982.
34. Lucas, J. N., Tenjin, T., Straume, T., Pinkel, D., Moore, D. D., Litt, M., and Gray, J. W. Rapid human chromosome aberration analysis using fluorescence *in situ* hybridization [published erratum appears in *Int. J. Radiat. Biol.*, 56 (Suppl. 2): 201, 1989]. *Int. J. Radiat. Biol.*, 56: 35-44, 1989.
35. Morton, N. E. Parameters of the human genome. *Proc. Natl. Acad. Sci. USA*, 88: 7474-7476, 1991.
36. Melo, J. V., Yan, X. H., Diamond, J., Lin, F., Cross, N. C., and Goldman, J. M. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of very small numbers of transcripts: the risk in misinterpreting negative results. *Leukemia (Baltimore)*, 10: 1217-1221, 1996.
37. Zhang, Y. H., and McCabe, E. R. RNA analysis from newborn screening dried blood specimens. *Hum. Genet.*, 89: 311-314, 1992.
38. Liu, Y., Hernandez, A. M., Shibata, D., and Cortopassi, G. A. BCL2 translocation frequency rises with age in humans. *Proc. Natl. Acad. Sci. USA*, 91: 8910-8914, 1994.
39. Biernaux, C., Loos, M., Sels, A., Huez, G., and Stryckmans, P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood*, 86: 3118-3122, 1995.
40. Chen, H., and Eastmond, D. A. Topoisomerase inhibition by phenolic metabolites: a potential mechanism for benzene's clastogenic effects. *Carcinogenesis (Lond)*, 16: 2301-2307, 1995.