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# BIOMARKERS IN THE MOLECULAR EPIDEMIOLOGY OF BENZENE-EXPOSED WORKERS

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Biomarkers can be classified into three categories: biomarkers of exposure, susceptibility, and early effect. Along with colleagues from the National Cancer Institute, the Chinese Academy of Preventive Medicine in Beijing, the Shanghai Anti-Epidemic Center, the University of North Carolina, and other institutions in the United States, we have applied various biomarker methods to samples obtained from workers exposed to high levels of benzene. The goal of these studies is to develop and validate (1) biomarkers of exposure to benzene, such as albumin or hemoglobin adducts; (2) molecular markers of susceptibility to benzene, such as inherited polymorphisms in enzymes involved in the metabolism of benzene; and (3) biomarkers of the early effects of benzene, including hematotoxicity (complete blood cell counts), gene mutations (glycophorin A), and chromosome aberrations detected by fluorescence in situ hybridization (FISH), G-banding, and the micronucleus assay. An introduction to these studies has been presented previously (Rothman et al., 1996a, 1996b; Smith & Rothman, 1998; Smith & Zhang, 1998), and only those findings pertaining to biomarkers of exposure and early effects are discussed here.

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## SAMPLE COLLECTION AND EXPOSURE ASSESSMENT

Biological samples were collected from 44 healthy workers currently exposed to benzene with minimal exposure to toluene and other aromatic solvents in Shanghai, China, in October 1992. The same number of healthy controls without current or previous occupational exposure to benzene were enrolled from factories in the same geographic area. Controls were frequency matched by gender and age (5-yr intervals). Exclusion criteria for all subjects were history of cancer, therapeutic radiation, chemotherapy, or current pregnancy. Each subject was administered a questionnaire by a trained interviewer. Data collected included age, gender, current and lifelong tobacco use, current alcohol consumption, medical history, and work history. Height and weight of each subject were measured and peripheral blood was obtained by phlebotomy.

Individual exposure was monitored by organic vapor passive dosimetry badges (3M number 3500, St. Paul, MN), which were worn by each worker for a full work shift on 5 separate days during the 1- to 2-wk period prior to phlebotomy. An 8-h time-weighted average (TWA) exposure was calculated for benzene as the geometric mean of the 5 air measurements. Cumulative exposure to benzene was calculated by multiplying historical time-specific exposure estimates by the duration worked. All exposure assessment was performed blinded with respect to the biomarker analysis.

## BIOMARKERS OF EXPOSURE

The median benzene air level among the exposed workers was 31 ppm as an 8-h TWA (range 1–328 ppm). Air-monitoring data were confirmed by measures of urinary benzene metabolites; phenol, muconic acid, catechol, and hydroquinone showed strong, positive correlations with air benzene levels, and were substantially higher in exposed workers compared to controls (Rothman et al., 1998). In addition, current benzene air levels were inversely correlated with the absolute lymphocyte count among exposed workers, consistent with previous reports that lymphocytes are particularly sensitive to benzene (Rothman et al., 1996a, 1996b).

The initial metabolite of benzene, benzene oxide (BO), reacts with cysteinyl residues in hemoglobin (Hb) and albumin (Alb) to form protein adducts (BO-Hb and BO-Alb), which are presumed to be specific biomarkers of exposure to benzene. With Dr. Stephen Rappaport's lab in Chapel Hill, NC, we analyzed BO-Hb in 43 of the exposed workers and 42 unexposed controls and BO-Alb in a subsample consisting of 19 workers and 19 controls (Yeowell-O'Connell et al., 1998). The adducts were analyzed by gas chromatography–mass spectrometry (GC-MS) following reaction of the protein with trifluoroacetic anhydride and methanesulfonic acid. When subjects were divided into controls ($n$ = 42) and workers exposed to <31 ppm ($n$ = 21) and >31 ppm ($n$ = 22) benzene, median BO-Hb levels were 32.0, 46.7, and 129 pmol/g globin, respectively (correlation with exposure:

Spearman $r = .67$, $p < .0001$). These results represent the first observation in humans that BO-Hb levels are significantly correlated with benzene exposure. Median BO-Alb levels in these 3 groups were 103 ($n = 19$), 351 ($n = 7$), and 2010 ($n = 12$) pmol/g Alb, respectively, also reflecting a significant correlation with exposure (Spearman $r = .90$, $p < .0001$). These results clearly affirm the use of both Hb and Alb adducts of BO as biomarkers of exposure to high levels of benzene.

## BIOMARKERS OF EARLY EFFECT FROM BENZENE EXPOSURE

A potential method of predicting who is most at risk for benzene-induced leukemia is to determine the extent of the genetic damage it produces in exposed individuals, using biomarkers of early effect. One means of assessing genetic damage is to measure mutations in specific genes, such as glycophorin A (GPA) (Compton et al., 1991; Jensen & Bigbee, 1996). An increased level of "gene-duplicating" mutations in GPA was found in the benzene-exposed workers (Rothman et al., 1995). Interestingly, this increased mutation frequency was correlated with cumulative exposure to benzene. Since cumulative exposure to benzene may correlate best with leukemia risk, the GPA assay appears to have potential as a biomarker of early biological effect for benzene and other leukemogens. The GPA assay has drawbacks, however. First, it is relatively insensitive: High benzene exposure (mean TWA at 72 ppm) only elevated the combined mutant frequency from 16.3 to 23.0 per million, a 41% increase (Rothman et al., 1995). Second, it can only be performed on GPA heterozygous (type MN) individuals, who statistically constitute only 50% of any given population under study. Thus, although the GPA assay can provide important mechanistic information, it may not be an ideal biomarker of early effect.

The most common means of detecting genetic damage has traditionally been conventional cytogenetics. Numerous publications have demonstrated a clear association between benzene exposure and increased levels of chromosome aberrations in peripheral blood cells. Since chromosome aberrations in peripheral blood lymphocytes have been shown to be associated with increased risk for overall cancer incidence (Hagmar et al., 1994), especially for increased mortality from hematological malignancies (Bonassi et al., 1995), it is possible that specific chromosome aberrations may provide even better markers of future leukemia risk.

## SPECIFIC CHROMOSOME ABERRATIONS IN LEUKEMIA

Specific chromosome aberrations are the hallmark of human leukemia (Hagemeijer & Grosveld, 1996). Aneuploidy, the loss or gain of specific chromosomes in AML and MDS (such as trisomy 8 and monosomy 5 and 7), is commonly observed as specific chromosome translocations, inversions, and deletions [e.g., t(8;21), t(9;22), inv(16), and del(5q)] (Hagemeijer

& Grosveld, 1996). The loss of chromosomes 5 and 7 and their long-arm deletions are the two most common changes in therapy-related AML and MDS (t-AML and t-MDS), especially among patients previously treated with alkylating agents (Pedersen-Bjergaard et al., 1995). Treatment with topoisomerase II inhibitors is associated with balanced chromosome aberrations such as t(8;21) and t(11q23) in t-AML (Pedersen-Bjergaard et al., 1995). These specific chromosome aberrations are also more common among leukemia patients with previous exposure to chemical solvents (including chronic exposure to benzene, insecticides, petroleum, etc.) (Crane et al., 1996; Mitelman et al., 1981).

## DETECTION OF SPECIFIC CHROMOSOME ABERRATIONS BY FISH

We have applied FISH to determine the presence of specific chromosome aberrations in the lymphocytes of otherwise healthy workers exposed to benzene and matched controls. Initially, we studied hyperdiploidy levels of chromosome 9 in interphase cells because trisomy 9 has been observed in benzene-poisoned patients (Erdogan & Aksoy, 1973; Forni & Moreo, 1967) and benzene metabolites induce hyperdiploidy of this chromosome in cultured lymphocytes in vitro (Eastmond et al., 1994; Zhang et al., 1994). High benzene exposure was shown to increase hyperdiploidy of chromosome 9 in the lymphocytes of otherwise healthy workers, with trisomy 9 being the most prevalent form (Zhang et al., 1996). We have gone on to use interphase cytogenetics to study the hyperdiploidy of chromosomes 7 and 8, and these findings will be published shortly. Interphase cytogenetics cannot be used, however, to detect monosomy or rare translocations because of artifacts related to probe overlap (Eastmond & Pinkel, 1990). Monosomy of chromosomes 5 and 7 and translocation (8;21) are among the most common aberrations observed in AML. We therefore used chromosome painting and region-specific fluorescent probes to examine AML-specific aberrations, including –5, –7, del (5q31), del (7q22-34), and t(8;21), in metaphase spreads prepared from the lymphocytes of workers exposed to benzene and matched controls (Smith et al., 1998; Zhang et al., 1998a). We painted chromosomes 8 and 21 in lymphocyte metaphases from the 44 workers exposed to benzene and 44 matched controls. To examine dose-response relationships, the workers were divided into 2 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 chromosome 8 (1.2, 1.5, 2.4 per 100 metaphases; $p$ trend $< .0001$) and 21 (0.9, 1.1, 1.9; $p$ trend $< .0001$). Translocations between chromosomes 8 and 21 were increased up to 15-fold in highly exposed workers (0.01, 0.04, 0.16; $p$ trend $< .0001$). In one highly exposed individual these translocations were reciprocal and were detectable by reverse-transcription polymerase chain reaction (PCR) (Smith et al., 1998).

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 from benzene exposure.

We also used a novel FISH procedure to determine if specific aberrations in chromosomes 1, 5, and 7 occurred at an elevated rate in metaphase spreads prepared from the lymphocytes of the same benzene-exposed Chinese workers (Zhang et al., 1998a). We found that benzene exposure was associated with increases in the rates of monosomy 5 and 7 but not monosomy 1 ($p < .001$, $< .0001$, and $< .94$, respectively) and with increases in trisomy and tetrasomy frequencies of all three chromosomes. Long arm deletion of chromosomes 5 and 7 was increased in a dose-dependent fashion ($p = .014$ and $< .0001$) up to 3.5-fold in the exposed workers. These results demonstrate that leukemia-specific changes in chromosomes 5 and 7 can be detected by FISH in the peripheral blood of otherwise healthy benzene-exposed workers. We have also shown that benzene metabolites induce these same changes in vitro (Zhang et al., 1998b). Taken together, all of these data obtained using FISH suggest that aberrations in chromosomes 5, 7, 8, and 21 may be a useful biomarker of early biological effect for benzene exposure. Indeed, Dr. Luoping Zhang of Dr. Smith's laboratory has recently devised a FISH procedure to examine the leukemia-related changes in all four of these chromosomes simultaneously.

Studies are being planned to apply these methods in workers exposed to a broad range of benzene concentrations. In addition, we will explore the impact of interindividual variation in genes that activate and detoxify benzene and its metabolites on these outcomes, following up our previous report that variation in CYP2E1 and NQO1 influence the risk of benzene hematotoxicity (Rothman et al., 1997).

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