Measurement of DNA Repair Deficiency in Workers Exposed to Benzene

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We hypothesize that chronic exposure to environmental toxicants can induce genetic damage causing DNA repair deficiencies and leading to the postulated mutator phenotype of carcinogenesis. To test our hypothesis, a host cell reactivation (HCR) assay was used in which pCMVcat plasmids were damaged with UV light (175, 350 J/m² UV light), inactivating the chloramphenicol acetyltransferase reporter gene, and then transfected into lymphocytes. Transfected lymphocytes were therefore challenged to repair the damaged plasmids, reactivating the reporter gene. Xeroderma pigmentosum (XP) and Gaucher cell lines were used as positive and negative controls for the HCR assay. The Gaucher cell line repaired normally but XP cell lines demonstrated lower repair activity. Additionally, the repair activity of the XP heterozygous cell line showed intermediate repair compared to the homozygous XP and Gaucher cells. We used HCR to measure the effects of benzene exposure on 12 exposed and 8 nonexposed workers from a local benzene plant. Plasmids 175 J/m² and 350 J/m² were repaired with a mean frequency of 66% and 58%, respectively, in control workers compared to 71% and 62% in exposed workers. Conversely, more of the exposed workers were grouped into the reduced repair category than controls. These differences in repair capacity between exposed and control workers were, however, not statistically significant. The lack of significant differences between the exposed and control groups may be due to extremely low exposure to benzene (<0.3 ppm), small population size, or a lack of benzene genotoxicity at these concentrations. These results are consistent with a parallel hprt gene mutation assay. — Environ Health Perspect 104(Suppl 3):529–534 (1996)

Key words: DNA repair, benzene, host cell reactivation assay, xeroderma pigmentosum, occupational exposure

Introduction

In today’s industrialized environment, approximately 70,000 chemicals are in use (1). Five hundred new chemicals are introduced commercially each year resulting in a 37% increase in new chemicals over the past 7 years (2,3). In the United States, 40 possible carcinogens have been identified in drinking water and 60 possible carcinogens are being released into the air (4).

These chemicals can produce damage to DNA through adduct formation, alkylation, and strand breaks, resulting in mutations that are part of the multistep process of carcinogenesis (5,6).

An individual’s health risk from exposure to environmental toxicants is controlled by several host susceptibility factors (4,7). Individual variations in these susceptibility factors are based on inheritance of mutant genes and environmental influences. One such factor is based on individual variations in DNA repair capacity, which is most likely a primary determining element for long-term health problems. Patients with inherited DNA repair deficiencies, such as xeroderma pigmentosum (XP) and ataxia telangiectasia (AT), serve as model systems to document this phenomenon. XP individuals exhibit a reduced capacity to repair UV light-induced damage and are at a 2000-fold greater risk for the development of skin cancers (4,8). AT individuals show reduced ability to repair ionizing radiation-induced damage and have an increased risk for the development of lymphomas (9). The role that environmental factors have on individual DNA repair capacity is not fully elucidated and has been the focus of recent investigations. Oesch and Klein (10) have demonstrated a reduction in O6-alkylguanine alkyltransferase activity in tire storage workers exposed to ambient nitroso compounds found in tire storage areas and in clinical workers preparing cancer chemotherapeutic agents. In another study, Oesch et al. (11) examined the repair of ethenedenedine lesions in workers exposed to vinyl chloride. Though their findings were equivocal, Oesch and Klein were unable to rule out the possibility of an increased risk for cancer in this population. We have reported that populations exposed to environmental toxicants have defective DNA repair responses. Au et al. (12) observed higher chromosomal translocation rates in smokers versus nonsmokers when their lymphocytes were challenged to repair X-irradiation-induced damage. This indicates that smokers were unable to repair their radiation-damaged DNA as efficiently as nonsmokers. These findings are consistent with the findings of Celotti et al. (13) and Mayer et al. (14) who observed altered unscheduled DNA synthesis in chemically challenged lymphocytes of smokers. Workers exposed to butadiene demonstrated abnormal DNA repair response when challenged with γ-irradiation, and this DNA repair abnormality was significantly correlated with butadiene urine metabolites (15). We have also reported that residents living near uranium mining sites exhibit significantly higher chromosome aberrations than the controls when their lymphocytes were challenged to repair γ-ray-induced DNA strand breaks (16). The defective DNA repair
responses observed in these studies support the existence of a mutator phenotype.

The mutator phenotype phenomenon was originally proposed by Loeb (5) and substantiated by Chang and Little (17) in experiments in which persistent and elevated spontaneous mutation levels were observed in Chinese hamster ovary clonal progeny whose parental cells had survived irradiation. In addition, Loeb (18) and Modrich (19) also observed a similar genetic instability in tumor cells from individuals with hereditary nonpolyposis colorectal cancer (HNCC) and in a subset of nonhereditary colorectal cancer patients (CRC). Patients with HNCC have inherited one normal and one mutant mismatch repair gene allele. Normal cells from these patients do not display genetic instability or any measurable mismatch repair deficiency (19-21); however, in the neoplastic cells from HNCC patients, the wild type allele has been lost or mutated through intrinsic or environmental factors, resulting in genomic instability. But in the case of nonhereditary CRC, genomic instability may be entirely attributed to environmentally induced defective mismatch repair since CRC patients presumably began with both wild type alleles. These observations point out that exposure to environmental toxicants can damage DNA and affect DNA repair efficiency and fidelity.

In the present study, the host cell reactivation (HCR) assay was selected to measure individual DNA repair capacity in benzene-exposed workers. Benzene is a common environmental pollutant found all over the world. The major sources of benzene exposure come from petroleum and petroleum products (22). As early as 1928, benzene has been implicated as a leukemogen (23). Current concern is whether exposure to reduced concentrations of benzene may still be hazardous to workers. Athas et al. (24) and Wei et al. (25,26) have validated the HCR assay by determination of DNA repair capacity in XP cell lines and XP patients and in patients with basal cell carcinoma. Based on these studies, we hypothesize that the HCR assay can document the reduction of DNA repair capacity in occupationally exposed populations. HCR measures DNA repair capacity as a total process in which not only accurate DNA repair is measured but also the cell’s ability to express the restored gene into a functional protein product. In short, a UV-light-irradiated plasmid (pCMVcat) containing a damaged chloramphenicol acetyltransferase reporter gene (CAT) is transfected into host lymphocytes. The host cells are challenged to repair the damaged plasmid. If the lesions are correctly repaired, the cells will express the novel CAT protein and CAT activity is directly related to repair of the plasmid.

**Materials and Methods**

**Selection of Study Population**

All subjects and controls were selected from petrochemical workers from a local benzene manufacturing plant in Texas. Work sites were identified as high or low based on whether or not benzene was handled. Workers from these areas were recruited as exposed and matched nonexposed workers. The workers were asked to fill out questionnaires documenting tobacco use and radiation and chemical exposures during work activities. Workers who were nonsmokers and who had no exposure to other toxicants were selected. These selected workers were asked to sign consent forms, to wear exposure monitors, and to provide blood samples at the end of the work shift.

**Lymphocyte Cultures**

Whole blood from benzene-exposed and -nonexposed workers was collected by venous puncture for isolation of lymphocytes using Ficol histopaque. Lymphocytes were initiated in RPMI 1640 medium supplemented with 15% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 1% L-glutamine, 1% penicillin/streptomycin, and 2% phytohemagglutinin and cultured in T-75 flasks at 37°C and 5% CO2.

**Xeroderma and Gaucher Cell Cultures**

One Gaucher cell line (GMO-1031) and three XP cell lines (GMO-4237, GMO-2345, and GMO-2246) were chosen for use as positive and negative controls to validate the activity of the HCR assay. GMO-2345 (+/−), a homozygous XP-C cell line, is highly DNA repair deficient; GMO-2246 (+/−) also is homozygous, but it is an XP-C cell line that is moderately DNA repair deficient. GMO-4237 (+/+), a heterozygous XP-C cell line with relatively normal repair and the Gaucher GMO-1031 cell line is apparently normal (Table 1). All cell lines were purchased from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ. Cells were grown without antibiotics in RPMI 1640 (Gibco, New York, NY) supplemented with 15% heat-inactivated fetal bovine serum (Irvine Scientific) and 1% L-glutamine in T-75 flasks at 37°C and 5% CO2.

**XP Survival Assay**

XP cells were collected from cultures by centrifugation at 800 rpm for 10 min at 25°C (Beckman GS-6R; Beckman Instruments, Schaumberg, IL). The media supernatant was decanted and saved. The cells were washed twice with 0.25 M Tris-buffered saline (TBS), pH 7.4, and placed into 100×15mm petri dishes (2x10^6 cells/2 ml TBS). Aliquots of cells were irradiated with UV light at doses of 10, 25, 50, and 100 J/m2. The cells were then collected and cultured in T-75 flasks using the reserved media plus fresh media (RPMI 1640, 15% FBS, 1% L-glutamine): 1:1. After 48 hr the cells were once again collected by centrifugation, samples were taken, and total cell number was determined using a hemocytometer.

**pCMVcat Plasmid**

We used the pCMVcat plasmid (a gift from L. Grossman, Johns Hopkins University) derived from cytomegalovirus and containing an inserted CAT gene. The reporter gene produces a unique protein product not normally found in eukaryotic cells. pCMVcat plasmid was isolated from *Escherichia coli* HB101 bacteria using alkaline Triton X-100 lysis and purified by CsCl density gradient centrifugation. This produces pCMVcat plasmid that is greater than 90% pure. Aliquots of purified plasmid were irradiated with 175, 350, and 700 J/m2 UV254nm to induce thymine dimers that inactivated the CAT gene (22).

**Transfection**

After 60 hr of culture, cells were centrifuged and the supernatant was removed, filtered, and saved. Cells were washed twice with TBS (0.25 M, pH 7.4) and then resuspended in TBS at a concentration of 8x10^6 cell/ml. Two hundred fifty micro liters of cell suspension (2x10^6 cells) was added to 50 μl of transfection mixture (5 μl plasmid DNA [50 μg/μl], 12.5 μl diethylaminoethyl [DEAE]/Dextran [10 mg/μl]),
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32.5 ml TBS) and incubated for 15 min at room temperature in a hood. Transfection was stopped with the addition of 1 ml of the previously reserved supernatant; the transfectants were then centrifuged and the supernatant was removed by aspiration. The cells were resuspended and cultured with 1:1 reserved supernatant plus new media. The cultures were incubated at 37°C and 5% CO₂ for 40 hr prior to harvest (23).

**Cell Extracts**

Lymphocyte cultures from exposed and control groups were placed on ice and media was removed by aspiration. Two milliliters of incomplete TBS (0.25 M, pH 7.4, lacking Ca²⁺ and Mg²⁺) was used to wash and resuspend cells, and a sample was removed and used to determine cell concentration. Suspended cells were centrifuged (100 × g, for 10 min at 4°C) and the supernatant was aspirated. The cells were resuspended in 1 ml incomplete TBS and transferred to Eppendorf tubes. The Eppendorf tubes were centrifuged (4,000 rpm for 10 min at 4°C) and the supernatant was aspirated. Cells were resuspended to be lysed in 100 μl ice-cold Tris/EDTA (0.25 M Tris–HCl, 5 mM EDTA [ethylendiaminetetraacetic acid], pH 7.8). The cell suspension was subjected to three rounds of freeze–thaw and vortexing and then stored at −70°C prior to the CAT assay.

XP and Gaucher cell extracts were similarly prepared, except that no samples were taken to count and cells were lysed using reporter lysis buffer from Promega (Madison, WI). This was followed by one cycle of freeze–thaw prior to centrifugation at 16,000 × g for 5 min. Cleared cell extracts were then quick frozen and stored at −70°C.

**CAT Assay**

Cell extracts from benzene-exposed workers and their controls, which had been stored at −70°C, were placed in a 65°C water bath for 10 min. The samples were centrifuged in an Eppendorf centrifuge for 10 min at 16,000 × g. Fifty microliters of cleared cell extract was placed in scintillation vials and 25 μl of reaction mixture (7.5 μl of 5 mM chloramphenicol, 1.0 μl of 2.5 mM 3H-acetyl coenzyme A, and 16.5 μl of D₂O) was added. Four milliliters of scintillation cocktail (Betamax II Scintillation fluid, water immiscible; ICN Biomedical, Costa Mesa, CA) was layered on top of the reaction mixture and the vials were counted for 10 cycles.

**Results**

To verify the identity of the XP cell lines, a survival experiment was initiated. Survival was based on competence of the cell line to repair UV light-induced damage. XP cell lines GMO-2345, GMO-2246, and GMO-4237 were subjected to 10, 25, 50, and 100 J/m² UV-light irradiation. Figure 1 summarizes the survival results in which all XP cell lines behaved phenotypically in accordance with their genotype and XP complementation grouping. The XP cell line GMO-4237, which is derived from a clinically normal female and has a heterozygous genotype, is from the XP-C complementation group. This cell line demonstrated the highest survival level at all UV-light doses. GMO-2246 is also from a complementation group XP-C female and has a homozygous genotype. UV-light damage, at all doses in this cell line, is repaired at an intermediate level between GMO-4237 and GMO-2345.

XP-4237 is derived from a severely affected complementation group, XP-A, female and was found to have the lowest survival over all UV-light doses.

XP and Gaucher cell lines served as positive and negative controls, respectively, in our experiments. Figure 2 summarizes...
the DNA repair capacities found in these cell lines. DNA repair capacity correlated well according to their complementation groups and genotype. The apparent normal Gaucher cell line (GM0-1031) was able to repair 90% of the damaged induced by 350 J/m² UV light and 45% of the damage induced by 700 J/m². The heterozygous XP cell line GMO-4237 repaired 60% (± 17.6 SE) and 25% (± 3.1 SE) of the damage, respectively. The XP cell line GMO-2246 repaired the identical damage at even lower levels (350 J/m²—38% ± 2.8 SE; 700 J/m²—22% ± 4.2 SE) while GMO-2345 repaired the damage at the lowest levels (350 J/m²—8% ± 1.5 SE; 700 J/m²—2% ± 1.3 SE).

The benzene study population is described in Table 2. Both exposed and control groups were adequately matched. The exposed population consisted of 12 males (86%) and 2 females (14%) and the control group consisted of 8 males (89%) and 1 female (11%). We were unable to recruit additional females to increase the sample size. To prevent confounding by sexual differences, the females were removed from our data for further analysis. All remaining exposed and control subjects were male nonsmokers having a mean age of 46.3 years (± 6.5 years) and 49.5 years (± 10.6 years) respectively. The benzene exposure doses for even the exposed workers were below the detection limit of the personal monitors (≤ 0.3 ppm).

Cells from both exposed and control populations were unable to repair 100% of the dimers on the plasmids. To analyze this, all data from both the exposed and control populations were pooled (n = 20). The mean level of repair in the pooled data for plasmid 350 J/m² was 60.26% (CI, 6.2) and for plasmid 175 J/m² was 68.79% (CI, 4.8). We determined a cutoff point for reduced repair to be the mean of repair for the plasmid less its confidence interval. Consequently, the cutoff for plasmid 350 J/m² was 54% and for plasmid 175 J/m² was 64%. Using these criteria, the pooled data were then ranked as normal repair (above the cutoff) or reduced repair (below the cutoff) (see Figure 3). Those individuals with reduced repair were significantly different from those showing normal repair (p ≤ 0.001 350 J/m²; p ≤ 0.001 175 J/m²).

More importantly, those individuals classified as repair deficient using plasmid 175 J/m² were also identified as repair deficient using plasmid 350 J/m² for both the exposed and control groups. Another analysis of the data compares the repair activities of the exposed population with the control population (Figure 4). The control group repaired 66% (± 11.8 SD) of the damage in plasmid 175 J/m² compared to 71% (± 10.5 SD) of the damage repaired by the exposed group. The control group repaired 58% (± 13.3 SD) of the damage in plasmid 350 J/m² compared to 62% (± 15 SD) of the damage repaired by the exposed group. Interindividual variations in repair capacity are apparent in both populations.

**Discussion**

The HCR assay is being used to identify individuals who are at increased health risk due to a reduction in DNA repair capacity. We hypothesize that DNA repair capacity is influenced by exposure to chemical toxicants. To this end, we have conducted a series of experiments to verify the sensitivity of the HCR assay and to establish XP and Gaucher cell lines as negative and positive controls for our study. A small group of benzene-exposed workers was used as our target population. Experiments with XP and Gaucher cell lines attest to the sensitivity of the HCR assay: the assay is able to distinguish between the normal repair capacity observed with the Gaucher GM0-1031 cell line from the relatively normal repair capacity found in the heterozygous XP-C (GMO-4237) cell line (Figure 2). The homozygous XP-A (GMO-2345) and XP-C (GMO-2246) cell lines were identified as having reduced DNA repair capacity. These results are in accordance with those of Athas et al. (24) who reported that the XP-A (GMO-2345) cell line repaired only 3% of the damage in plasmid 300 J/m² while the XP-C (GMO-2246) cell line repaired 22% of the damage. These cell lines therefore provide the necessary positive and negative controls for our experiments.

To test our hypothesis on the influence of chemical toxicants on DNA repair capacity, we recruited a group of benzene-exposed workers and controls. We were able to identify individuals from both exposed and control populations exhibiting a reduced capacity to repair DNA damage (Figure 4). These results are not unexpected because we anticipated a normal distribution of DNA repair capacity in the general population, but the importance of being able to identify these individuals should be emphasized because we feel that this subset of the general population is at increased health risk due to a reduced ability to repair DNA damage when exposed to chemical toxicants.
The mean repair capacity between exposed and control populations was not significantly different. Both populations demonstrated large individual variations in repair capacity; this may explain why no significance could be found. In the study by Oesch et al. (11), significant individual variation in the repair of etheno adenine lesions was also observed. Because of this individual variation, the population size must be increased to find possible significant differences in repair capacity. Additionally, use of this study will enable a better estimation of what is required for future studies. It is also possible that, at this benzene concentration, this chemical may only be minimally genotoxic as shown in the reduced DNA repair capacity observed in some exposed workers.

In a parallel study, no increase in the mutation frequency of the hprt gene in the exposed workers compared to the control worker population was documented (JB Ward, personal communication). This is consistent with our observations.

We have demonstrated the application of the HCR assay as a tool to monitor workers for DNA repair deficiencies for the first time. Our data suggest that exposure to ≤0.3 ppm benzene may only be minimally genotoxic. In addition, significant interindividual variations were demonstrated by this exceptionally sensitive assay, suggesting that other factors such as polymorphism for metabolism of chemicals may also play a role. Studies using large sample sizes and workers exposed to other toxicants are ongoing to validate and extend our observations.

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