Monitoring Populations for DNA Repair Deficiency and for Cancer Susceptibility

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The induction of a mutator phenotype has been hypothesized to cause the accumulation of multiple mutations in the development of cancer. Recent evidence suggests that the mutator phenotype is associated with DNA repair deficiencies. We have been using a challenge assay to study exposed populations to test our hypothesis that exposure to environmental toxicants can induce DNA repair deficiency in somatic cells. In this assay, lymphocytes were irradiated in vitro to challenge cells to repair the radiation-induced DNA strand breaks. An increase of chromosome aberrations in the challenged cells from toxicant-exposed populations compared to nonexposed populations is used to indicate abnormal DNA repair response. From studies of cigarette smokers, butadiene-exposed workers, and uranium-exposed residents, the assay showed that these exposed populations had mutagen-induced abnormal DNA repair response. The phenomenon was also demonstrated using experimental animals. Mice were exposed in vivo to two different doses of N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) and their lymphocytes were challenged with one dose of a radiomimetic chemical, bleomycin, in vitro. These challenged lymphocytes showed an MNNG dose-dependent increase of abnormal DNA repair response. In a population that was potentially exposed to teratogens—mothers having children with neural tube defects— lymphocytes from these mothers did not have the abnormal response in our assay. In studies with patients, we reported that lymphocytes from Down's syndrome patients have the abnormal DNA repair response. Lymphocytes from skin cancer-prone patients (epidermodysplasia verruci-formis) have normal response to γ-ray challenge but abnormal response to UV-light challenge. These patient studies also indicate that the challenge assay is useful in documenting the radiosensitivity of Down's syndrome and the UV sensitivity in EV patients. In most cases, the challenge assay is more sensitive in detecting biological effects than the standard chromosome aberration assay. Our series of studies indicates that the challenge assay can be used to document biological effects from exposure to mutagens and that the effect is an abnormal DNA repair response. This abnormality can increase the risk for development of cancer. The repair deficiency is currently being validated using a plasmid transfection (host-reactivaton) assay. The need to integrate chromosome aberration and the challenge assays with other relevant assays for better documentation of biological effects and for more precise prediction of health risk will be presented. Our experience in using genetic polymorphism and host-reactivation assays will be discussed. — Environ Health Perspect 104(Suppl 3):579–584 (1996)

Key words: DNA repair, chromosome aberrations, environmental toxicology, predisposition, cancer susceptibility, genotoxicity

Introduction

There is increased interest in using biological markers to monitor populations for identification of excessive exposure to environmental toxicants. The ultimate goal is to use these biomarker data to indicate early disease and to predict increased risk for development of long-term health consequences. The choice of biomarkers for population studies will therefore determine the usefulness of the results in public health. In addition, the sensitivity of the assay should be taken into consideration. Some of the assays have been very useful and have had adequate sensitivity in documenting biological effects of exposed workers in the past; however, with improved work conditions through environmental engineering, current workers are usually exposed to smaller amounts of toxicants than before. Furthermore, through automation of industrial processes, fewer workers are employed in these industries. This may cause problems with studies that require substantial population sizes for analyses. With recent attention focused on hazardous waste sites, population studies are not limited to investigating workers but have been extended to the general public. Residents living around hazardous areas are assumed to be exposed to less toxicant than allowed at the occupational levels, based on air sampling data. In addition, the exposure conditions for the public are different from those for the workers. Therefore, studies involving the general public require additional considerations regarding experimental design and choice of biomarkers.

Chromosome aberration is one of the most extensively used biomarkers for population monitoring. It is often considered a gold standard for documentation of biological effects in exposed populations. Furthermore, unlike most other biomarkers, existence of chromosome aberrations has been shown to be associated with health effects. For example, chromosome aberration is frequently used as an internal biological dosimeter for exposure to ionizing radiations (1,2), and the dose–response relationship of chromosome aberration to exposure is similar to that for leukemia mortality (3). In a prospective cohort study, populations with increased chromosome aberrations have higher leukemia mortality than those with lower chromosome aberrations (4,5). Therefore, this biomarker can be used to predict health consequences. One drawback of this gold

standard assay is that it may not have the sensitivity to detect effects after exposure to very low doses of toxicants. For exposure to ionizing radiations, the detection limit is of the order of 10 cGy for recent exposure (6), but the detection limits for other biomarkers are mostly unknown. Therefore, for population studies, it is prudent to use the standard chromosome aberration assay in conjunction with newer assays such as the fluorescence in situ hybridization assay for detecting balanced chromosome translocations (7–9) and challenge assays to detect abnormal DNA repair response leading to the development of chromosome alterations.

Carcinogenesis is a multistage process involving multiple and sequential genetic alterations. It has been proposed that an initial step for the development of cancer involves the induction of a mutator phenotype (10). Recent evidence suggests that the phenomenon of mutator phenotype leading to genetic instability may be caused by DNA repair deficiency (11,12); this repair deficiency may be caused by exposure to environmental toxicants. For example, tire-storage workers exposed to nitrosocompounds and hospital personnel exposed to antineoplastic drugs were deficient in methylguanine alkyltransferase activities (13,14). Abnormal repair synthesis was documented in lymphocytes of cigarette smokers and drug addicts when their cells were challenged with UV light or chemicals (15–17). We have used a challenge assay to detect abnormal DNA repair response. In this assay, lymphocytes from toxicant-exposed and nonexposed individuals are irradiated during the G_1 phase in vitro with precise doses of ionizing radiation to induce DNA strand breaks; these cells are challenged to repair the radiation-induced damage. The assumption is that lymphocytes from toxicant-exposed individuals will have DNA repair deficiency due to modification of DNA/proteins (e.g., adducts) or mutation of DNA repair genes and will make more mistakes in repairing the DNA strand breaks than lymphocytes from controls. Since the radiation-induced DNA damage and repair occur during the G_1 phase as determined by our experimental protocol, cells from toxicant-exposed individuals will have more chromosome-type abnormalities than those from controls (18). The significance of DNA repair deficiency in the carcinogenic process is that such abnormality causes multiple and sequential genetic alterations in cells, thus promoting the evolution of genetic

changes that are relevant to the development of cancer.

The following is a description of our work using the challenge assay to detect abnormal DNA repair response in exposed populations. We will present the possibility of integrating several biomarkers in population studies to predict health risk.

Methods

Challenge Assay

The challenge assay was performed using lymphocytes from human populations and from mice. Blood cultures were set up according to standard procedures using RPMI medium (19,20). At 24 hr after initiation of cultures, cells were exposed to specific doses of radiation or to a radiomimetic chemical, bleomycin. Immediately after irradiation or treatment with chemical, bromodeoxyuridine was added to each culture (final concentration 10 μM) to label cells for identification of their proliferation patterns. These cultures were wrapped with aluminum foil to prevent exposure to light and were maintained in a humidified 5% CO_2 atmosphere and at 37°C. At 50 hr after initiation of human lymphocyte cultures, colcemid was added to each culture to arrest cells in mitosis. For mouse lymphocyte cultures, colcemid was added at 41 hr after initiation of cultures. One hr later, the cultures were harvested and slides were prepared. The slides were stained using the fluorescence-plus-Giemsa technique and viewed under a microscope to select cells having the first metaphase staining pattern for documentation of chromosome aberrations.

Results

Challenge Assay on Lymphocytes from Patients

Lymphocytes from six young Down's syndrome patients and six age-matched controls were investigated using the challenge assay (21). The unchallenged (background) frequencies of chromosome aberrations were the same for both groups of participants. After challenging the cells with 150 cGy X rays at the G_0 and G_1 phases of the cell cycles, the Down's syndrome cells had significantly more chromosome aberrations, thus abnormal DNA repair response, than the controls ($p < 0.02$). The percentages of aberrant cells from the G_0-irradiated cells were 34.3 ± 4.5 and 26.7 ± 9.0 for Down's syndrome and controls, respectively. The frequencies for the G_1-irradiated cells

were 36.2 ± 7.1 and 27.0 ± 8.8, respectively. Furthermore, the locations of the break sites were preferentially distributed at cancer break points and oncogene locations (22).

Epidermodysplasia verruciformis (EV) patients are known to have a very high incidence of sunlight-induced skin lesions. Lymphocytes from three EV patients and their matched controls were investigated using our challenge assay (23). It was found that these cells responded to γ-ray challenge like normal cells; however, when these cells were challenged with UV light (4 or 2 + 2 J/m²; the combined doses were each separated by 1 hr), the EV lymphocytes had significantly more chromosome aberrations than the controls ($p < 0.05$).

Challenge Assay Using Exposed Populations

Heavy cigarette smokers ($n = 12$) who had been consuming over 1 pack of cigarettes per day for over 10 years and matched nonsmokers ($n = 8$) were recruited for investigating the effect of cigarette smoking on DNA repair response (19). The background chromosome aberration frequencies were similar to each other for both groups of participants; however, when their lymphocytes were irradiated with 100 + 100 cGy γ rays (the two doses were separated by 1 hr), those from cigarette smokers had 1.35 times more dicentric frequencies than the nonsmokers (Figure 1) and the difference was significant at $p < 0.05$.

Ten butadiene-exposed workers and 10 matched nonexposed co-workers were recruited for our study (24). The mean exposure dose for the target population was 3.5 ppm and for the control it was 0.03 ppm. The background chromosome aberration frequency for the exposed population was higher than that for the control, but the difference was not significant. After the challenge with 100 cGy γ rays, the
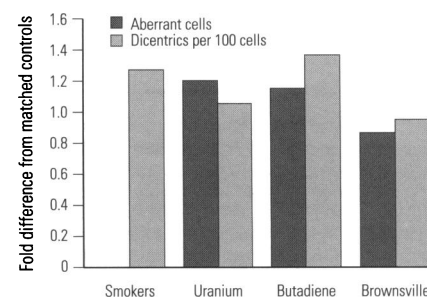

Figure 1. DNA repair response of exposed populations compared with matched controls based on challenge assay.

exposed group had a significantly higher percentage of aberrant cells and dicentric frequency per 100 cells than the controls ($p < 0.05$, Figure 1). In addition, the dicentric frequency for each exposed worker was significantly correlated with the concentration of a butadiene metabolite [1,2-dihydroxy-4(N-acetylcysteinyl-S)butane] in urine of these workers (correlation of coefficient of 0.6 with $p < 0.01$).

Residents living around uranium mining/milling sites are potentially exposed to mining waste. A study was conducted to investigate whether living in such an environment could have increased health risk. Twenty-four residents in the target area and 24 matched residents in a control area were recruited for our study (25). None of the participants worked in the uranium industry and none of them were cigarette smokers. The target residents were found to have a slightly higher frequency of background chromosome aberrations than the controls, although the difference was not significant. After their lymphocytes were challenged with 100 cGy γ rays, the target residents had a significantly higher percentage of aberrant cells than the controls ($p < 0.05$, Figure 1). In addition, the dicentric frequency of the target population was higher than that of the controls ($p < 0.25$).

From 1989 to 1991, a cluster of children with neural tube defects was detected in Brownsville, Texas. A study using the challenge assay was conducted on mothers with affected children to determine whether exposure to environmental mutagens could be a cause of the health problems (26). Nineteen of the targeted mothers were recruited and matched with 14 mothers who had normal children. Lymphocytes from these women were challenged with 100 cGy γ rays. Our study indicates that there was no difference in the background or the challenged chromosome aberration frequencies between the target and control mothers ($p > 0.5$, Figure 1).

Challenge Study with Experimental Animals

Experiments were conducted using mice to mimic human conditions to investigate whether *in vivo* exposure to mutagens would cause abnormal DNA repair response when their lymphocytes were challenged *in vitro* with a DNA-damaging agent. Male C57Bl/6 mice weighing 15 to 20 gm were intraperitoneally injected two times with 25 or 50 mg/Kg N-methyl-N'-nitro-N-nitroso guanidine (MNNG), a minimally clastogenic mutagen. The two doses were

separated by 24 hr. At 8 hr after the second injection, mice were sacrificed and lymphocytes were removed for establishment of cell cultures according to the procedure of Au et al. (20). At 24 hr after initiation of cell cultures, lymphocytes were exposed to 3 μg/ml bleomycin, a radiomimetic agent. Cells were harvested at 42 hr. Lymphocytes from untreated mice, mice treated with MNNG alone, and lymphocytes treated with bleomycin *in vitro* alone were used as controls. It was found that the frequency of chromosome aberrations from lymphocytes with the combined treatment was significantly higher than those expected, based on simple additive effects from each of the agents MNNG and bleomycin ($p < 0.01$; Figure 2).

Discussion

Using the challenge assay, we have shown that the radiosensitivity that has been documented in Down's syndrome patients may be caused by their abnormal DNA repair response to ionizing radiation. In another study, lymphocytes from EV patients had normal response to the same challenge by γ rays; however, they had abnormal response after exposure to UV light. This abnormality is consistent with EV patients' increased incidence of cutaneous cancer in sunlight-exposed skin. The data suggest that the challenge assay can be used to document abnormal DNA repair response.

Using cigarette smokers, butadiene-exposed workers, and uranium-exposed residents, we have shown that the challenge assay is capable of detecting excessive exposure to toxicants as indicated by their expression of abnormal DNA repair response (19,24,25). In particular, the abnormal response among butadiene-exposed workers was significantly correlated with a butadiene metabolite in the urine of these workers, indicating a cause–effect relationship (24). For butadiene-exposed workers and uranium-exposed residents, the spontaneous chromosome aberration frequencies of the exposed populations were higher than those from their matched controls, although the difference was not significant. This indicates that their exposures to toxicants may be too low to be detectable by the standard chromosome aberration assay. For the uranium-exposed residents, this indicates that their exposure to ionizing radiation was lower than the maximal permissible occupational doses or less than 10 cGy acute exposure to high-LET radiation (6). The results also indicate that the exposure doses were high enough to cause
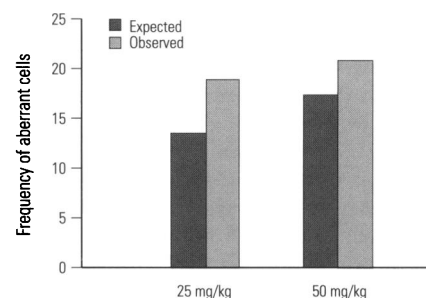

Figure 2. DNA repair response in mice exposed to N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) *in vivo* and then bleomycin *in vitro*.

abnormal DNA repair response and that the challenge assay is more sensitive than the standard chromosome aberration assay in detecting biological effects from exposure to toxicants. Although the mechanisms for the abnormal DNA repair response are not known, the abnormality may be caused by blockage of repair processes on DNA (e.g., adducts) or by mutation of genes that code for DNA repair enzymes (18). Because the DNA repair process involves multiple enzymes, it represents an enormous target for insult by toxicants. In addition, because many of the same repair enzymes serve on multiple repair pathways, we hypothesize that DNA repair defects caused by different toxicants can be detected using only γ-ray challenge. The abnormal DNA repair response will cause cells from affected individuals to make more mistakes in the repair of DNA damage, especially from further exposure to mutagens. The abnormality may cause these populations to have increased health risk.

In our study with mothers who had children with neural tube defects, no difference in chromosome aberrations or abnormal DNA repair response was detected in the affected mothers compared with the unaffected controls (26). This suggests that the affected mothers may not have been exposed excessively to environmental mutagens; however, their exposure to teratogens leading to the birth of children with malformations cannot be ruled out.

To characterize the abnormal DNA repair response further, we have been working on a host-reactivation assay to document DNA repair deficiency. This assay uses a pCMVcat plasmid containing a chloramphenicol acetyltransferase (CAT) reporter gene (27). The plasmids are irradiated with UV light to inactivate the reporter gene before transfection. After transfection, successful repair of the

UV-induced damage on the reporter gene by the host cells will allow expression of the gene. A unique feature of the CMVcat assay is that it measures the entire DNA repair process rather than one step of the process. We have confirmed that the assay is sensitive enough to distinguish the repair deficiency of heterozygous xeroderma pigmentosum patients from homozygotes and from nonxeroderma pigmentosum cells (28). In addition, the assay has been successfully field tested with lymphocytes from workers exposed to benzene (28). Although we observed reduced repair in cells from benzene-exposed workers compared with matched controls, the difference was not significant (28). The inability to detect the difference between the two groups may be because of the very low exposure concentrations (<0.3 ppm) to benzene for the exposed workers. Our observation is consistent with the lack of HPRT gene mutation in the same population (Ward, personal communication). Experiments using the assay on butadiene-exposed workers to further elucidate the sensitivity of this assay are ongoing.

For evaluation of health risk in exposed populations, variable individual/population response to toxicants must be taken into consideration. For example, it has been well documented that only approximately 10% of cigarette smokers develop lung cancer; therefore, there may be predisposed individuals in the population who make up a subpopulation with the highest risk. The ability to identify the predisposed individuals may be very helpful in reducing health effects in exposed populations. One of the mechanisms for predisposition is polymorphism in genes for metabolism of xenobiotics. With the advancement in molecular biology, individuals can be genotyped for polymorphism of several genes (29,30). We have been conducting genotyping studies to investigate the relationship between specific genetic polymorphisms and abnormal response to specific environmental toxicants as an indication of predisposition and increased health risk. This approach is used similarly by several investigators (31,32). We found that genetic polymorphism for the CYP2E1 gene is significantly high among cigarette-smoking
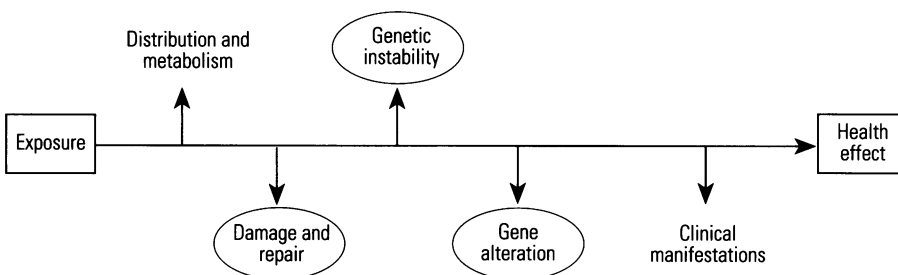

Figure 3. Flow diagram of biomarkers for health effects.

lung cancer patients and that their cells activated a cigarette smoke procarcinogen, 4-(methylnitroso amino)-1-(3-pyridyl)-1-butanone (NNK), to cause more sister chromatid exchanges than cells from nonsmokers (33).

Although the carcinogenic process is complex, a simple and common pathway seems to be involved for most cancers (11,34,35). In our laboratory we have used an experimental animal model to elucidate the process (36–38). In these studies, mammary cells from mice irradiated with a carcinogenic dose of X rays were established and investigated sequentially for genetic changes that lead to malignant transformation of the irradiated cells. The following sequential changes were observed. At the early stages the cultured cells were normal morphologically but had extensive chromosome instability, which was followed by the evolution of cells having a stabilized but abnormal karyotype. At this stage, the cells were not transformed and they did not form tumors upon injection into host mice. Later, the cells lost the expression of the retinoblastoma (Rb) gene, which was followed by the amplification of the c-myc protooncogene. At this stage, the cells that appeared transformed became malignant. The involvement of the Rb gene in the transformation process was confirmed by the transfection of normal Rb gene into the Rb-deficient and malignant cells. The transfected cells lost their malignant expression in host mice. Our studies (36–38), together with those from others, indicate that detection of biological effects which occur along the common pathway should allow us to identify the toxicant-induced health effects with confidence.

In our laboratory we have been integrating the relevant biomarkers into our research program for evaluation of health risk in exposed populations (Figure 3). The initial step of this process is the documentation of exposure for the target populations through environmental monitoring (39). This is followed by documentation of internal exposure (e.g., butadiene metabolites in urine of butadiene-exposed workers) (24,40). Under certain conditions, individuals with genetic polymorphism for metabolism and detoxification of xenobiotics may be at the highest risk for exposure to specific toxicants in an exposed population (33). Our assumption is that excessive exposure to toxicants will cause DNA damage, abnormality in DNA repair, and genetic instability. We intend to document these changes by using a variety of assays: chromosome aberration, challenge assays, and host reactivation assays. Finally, we intend to detect changes in specific genes (e.g., oncogenes and tumor suppressor genes) as an indication of health consequences.

In summary, the challenge assay is demonstrated to be useful in monitoring exposed populations for toxicant-induced abnormal DNA repair response. Based on our experimental data, we estimate that if we use a sample size of 12 per group, we should be able to detect a difference of 10% in dicentric frequency with statistical confidence. The assay should, however, be used with other relevent assays such as genetic polymorphism, host reactivation, gene mutation, and molecular genetic assays for better documentation of biological effects from exposure to toxicants and for more precise prediction of health risk.

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