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Project Summary

Evaluation of Exposure Markers

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A novel microgel electrophoresis assay has been developed for directly evaluating, in individual cells, the frequency of single strand DNA breaks and/or alkali-labile sites. This technique, called the single cell gel (SCG) electrophoresis assay, requires processing only a few hundred to a few thousand cells. The requirement for an extremely small number of cells makes it possible to evaluate the level and intercellular variability of DNA damage induced by genotoxic agents in virtually any eukaryote cell population.

The primary purpose of this research has been to determine the suitability of this technique for detecting DNA damage induced by potentially genotoxic pollutants either in cells sampled from various organs of rodents or in cells sampled from humans. In conducting this work, the focus of the research has been on: (1) evaluating the specificity and sensitivity of the technique by determining the magnitude and kinetics of DNA damage induced in cultured mammalian cells (e.g., mouse or human peripheral blood leukocytes, Chinese hamster ovary cells, rodent hepatocytes) by a variety of genotoxic and nongenotoxic chemicals; (2) developing appropriate methods for isolating individual cells from organs (e.g., blood, brain, liver, spleen, testis, bone marrow, lung) of rodents; (3) evaluating the kinetics of DNA damage induced in various organs of male mice by a representative environmental genotoxic pollutant; (4) examining the applicability of the assay to peripheral blood leukocytes obtained from humans exposed to genotoxic agents; and (5) comparing the levels of DNA damage in the organs of mice collected at an EPA Superfund site and a concurrent control site.

In many of these studies the induction of DNA damage was investigated using three representative environmental genotoxic pollutants—acrylamide, trichloroethylene and dimethylbenzanthracene. Based on the results obtained, this technique will provide, with greater sensitivity than any other method currently available, data on the induction and persistence of organ-specific levels of DNA damage resulting from environmental exposure to genotoxic pollutants.

This Project Summary was developed by EPA's Environmental Monitoring Systems Laboratory, Las Vegas, NV, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

One approach for assessing the possible environmental consequences of hazardous waste pollution involves the assessment of genotoxic damage, cytotoxic damage and other adverse health effects in sentinel organisms. In marine environments, sea urchins, mussels, benthic worms, and various species of fish have been used (or proposed for use) as organisms with which to monitor for adverse effects resulting from toxic pollution. In terrestrial

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environments, birds and plants, particularly the Tradescantia stamen hair system, have long been used to assess toxic levels of environmental pollution. More recently, interest has focused on mammalian species living in close proximity to man. Data have been published demonstrating the demographic impact of toxic wastes at Love Canal, New York, on resident meadow vole populations. Associations have been reported between proximity to industrial areas and increased levels of genotoxic damage in feral house mice. Recent research has reported an increased frequency of genotoxic damage among rodents collected at a hazardous waste site in New Jersey.

Techniques that permit the sensitive detection of DNA damage are useful in studies of toxicology and carcinogenesis. Since the effects of toxicants are often tissue and cell-type specific, it is important to develop techniques that can detect DNA damage in a variety of organs or, more importantly, in individual cells obtained from various organs. Currently, the three most commonly used *in vivo* methods for ascertaining the ability of chemicals to induce DNA damage involve the scoring of chromosomal aberrations, micronuclei and/or sister chromatid exchanges in proliferating cell populations; the detection of DNA repair synthesis (so-called unscheduled DNA synthesis or UDS) in individual cells; and the detection of single-strand DNA breaks and/or alkali labile sites in pooled cell populations.

While providing information about damage in individual cells, the cytogenetic techniques (chromosome aberrations, etc.) are of limited value because of the need for proliferating cell populations and because the DNA damage must be processed into microscopically visible lesions. The autoradiographic technique (for the detection of UDS) is based on the excision repair of DNA lesions, as demonstrated by the incorporation of tritiated thymidine into DNA repair sites. While providing information at the level of the individual cell, the technique is technically cumbersome and not all DNA lesions are repaired with equal facility. Biochemical techniques to evaluate DNA damage directly (e.g., DNA strand breaks), such as alkaline elution or alkaline gel electrophoresis, appear to circumvent some of the problems associated with the other two techniques. However, the use of pooled cells eliminates an evaluation of damage in small target tissues and ignores the importance of intercellular differences in response.

Biochemical approaches for detecting DNA damage directly in single cells have been developed but have not been applied formally to *in vivo* research. DNA damage may now be directly quantitated in individual cells by lysing cells embedded in agarose on slides under mild alkaline conditions to allow the partial unwinding of DNA. To improve sensitivity for detecting DNA damage in isolated cells, a microgel electrophoresis technique has been developed in which cells are embedded in agarose gel on microscope slides, lysed by detergents and high salt and then electrophoresed under neutral conditions. Cells with increased DNA damage display increased migration of DNA from the nucleus towards the anode. The migrating DNA is quantitated by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. While the neutral conditions for lysis and electrophoresis permit the detection of double-strand DNA breaks, they do not allow for the detection of either single-strand breaks or alkali-labile sites. Since many agents induce from 5 to 2000 fold more single-strand breaks than double-strand breaks, neutral conditions are clearly not as sensitive as alkaline conditions in detecting DNA damage.

Recently, a microgel electrophoretic assay has been introduced which is capable of detecting DNA single-strand breaks and/or alkali-labile sites in individual cells. The importance of this assay lies in its ability to detect intercellular differences in DNA damage/repair and in the requirement for extremely small cell samples. Furthermore, this single cell gel (SCG) technique appears to be quite sensitive, being capable of detecting on the order of 250 single-strand breaks and/or alkali-labile sites in the DNA of a single cell. While not all DNA lesions are alkali-labile, nor do all lesions result in visible cytogenetic damage, many classes of lesions are revealed by this technique.

The results reported here center on the evaluation of the SCG assay for use as a primary approach for detecting the possible exposure of mammalian organisms to genotoxic pollutants. This work has included experiments to develop and characterize the assay and data obtained from studies to explore the sensitivity of the assay for detecting genotoxic damage induced *in vitro* and *in vivo*. In many of these experiments, specific attention has been paid to the ability of acrylamide, dimethylbenzanthracene and trichloroethylene, three representative environmental pollutants, to induce single-strand DNA breaks and/or alkali-labile sites in the DNA of mammalian cells. The principal purpose of this research has been to expand the application of the SCG assay to the detection of DNA damage induced by chemicals in mammalian cells *in vitro* and *in vivo* and ultimately to the assessment of genotoxic damage in resident free-living animals or in humans environmentally exposed to hazardous pollutants.

Procedure

The Basic SCG Technique

Up to 10,000 cells of a cell suspension are mixed with 75 µl of 0.5% low melting-point agarose at 37°C and then placed on a precleaned, fully-frosted microscope slide previously coated with 0.5% regular agarose. The cell suspension is immediately covered with a #1 coverglass and the slides kept at 4°C for 5 minutes to allow solidification of the agarose. After adding a third layer of low-melting agarose, and allowing for solidification, the slides are immersed in a lysing solution at 4°C for 1 hour to lyse the cells. The slides are then removed from the lysing solution and placed on a horizontal gel electrophoresis unit.

The unit is filled with fresh electrophoretic buffer to a level 0.25 cm above the slides. The slides are left in this high pH buffer for 20 minutes to allow unwinding of the DNA. This is followed by electrophoresis for 10 to 40 minutes at 25 volts. After electrophoresis, the slides are rinsed gently, to remove alkali and detergents that would interfere with ethidium bromide staining, by flooding them slowly with 0.4 M Tris, pH 7.5. After three 5-minute rinses, the slides are stained by placing 50-75 µl of a 10 µg/ml ethidium bromide solution in distilled water on each slide and covering the slide with a coverglass. Observations are made using a Zeiss fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm.

The Image Analysis System

After compariing various image analyzing systems, the Cambridge Instrument's Quantimet 520* image analyzer was selected for use. The Quantimet 520 consists of a gated CCD camera attached to the fluorescent microscope and wired into the image analysis hardware. The hardware is in turn attached to a graphics monitor for visualization of the digitized image, a mouse-controlled digitablet for editing the image, a dot matrix printer, and a Zenith 386 PC with a separate graphics monitor for running the Cambridge software.

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

The Cambridge software allows for the setting of brightness and contrast levels, saving the image in memory, setting image intensity detections thresholds, editing and/or amending the image, calibrating to relative units, and finally measuring the migration length electronically. This process, although far better than that used initially, was still time-consuming. To streamline the cell measurement process, a program in QBASIC (a Cambridge modification of the BASIC programming language) was written.

With the current version of this program, scoring time has been reduced to approximately 30 seconds per cell (10-15 minutes per slide if 25 cells are scored). A spreadsheet template has been prepared which imports the data file, calculates means and standard errors for each slide, evaluates frequency distributions, and presents the data in tabular form. A graphics software package is then used to create the line and bar graphs for presentations.

Results and Discussion

In Vitro Experiments

A series of *in vitro* studies was conducted to investigate the applicability of the SCG assay to the detection of chemically induced DNA damage in mammalian cells treated *in vitro* with a variety of DNA damaging agents. In these experiments human leukocytes, mouse leukocytes, Chinese hamster ovary cells, and mouse and rat hepatocytes were exposed to graded doses of several genotoxic chemicals.

The first series of experiments with human leukocytes was conducted to examine the differential ability of hydrogen peroxide to induce damage in the DNA of intact cells vs the DNA of cells after lysis. At the levels tested, hydrogen peroxide induced a significant increase in the migration of DNA, regardless of whether metabolically active cells or lysed cells were treated. However, the extent of DNA migration appeared much greater for lysed cells than for intact cells.

A second set of experiments examined the effect of exposure duration on DNA migration length in ficoll-hypaque isolated mouse leukocytes exposed to dimethylbenzanthracene (DMBA) and acrylamide (ACR). Exposure for 4-hour periods to DMBA resulted in increases in DNA migration that were not dose dependent, while similar exposures to ACR produced negative results. However, when mouse leukocytes were incubated in complete medium at 37°C in the presence of 1000 µM ACR for 30 min or less, a significant increase in DNA damage resulted; by 1 to 2 hours the extent of DNA migration was returning to control levels. Thus, initial experiments were negative because long sample times permitted sufficient time for DNA repair to remove the damage. It was determined that adding cytosine arabinoside (ARA-C), a DNA synthesis chain terminator, could be used to prevent ligation of repair sites during unscheduled DNA synthesis.

Chinese hamster ovary cells were used in a series of experiments to evaluate the response to ACR (a direct acting agent), and to trichloroethylene (TCE) and DMBA (agents requiring metabolic activation). The presence of S9 was required before an increase in DNA migration could be demonstrated following exposure to DMBA and TCE, and a positive dose response was seen with S9 present. Response to ACR was generally greater when S9 was present, and a positive dose response was demonstrated both with and without S9, the response being steeper with S9.

Migration patterns were more heterogeneous for cells exposed to TCE and DMBA than for those exposed to ACR. One explanation for this may be that individual cells vary in their permeability to the S9-dependent active metabolite(s) of TCE and DMBA. The overall results of these experiments are consistent with ACR and a metabolite of ACR having genotoxic activity, and with TCE and DMBA requiring metabolic activation to reactive forms.

The final set of *in vitro* experiments adapted the rodent hepatocyte assay to SCG procedures. Mouse hepatocytes were freshly isolated for each test. Resulting cultures were exposed to two doses of cyclophosphamide (CP), a well-known alkylating agent requiring metabolic activation. Parenchymal cells (which possess the capability for metabolic activation) demonstrated significant increases in the lengths of DNA migration; furthermore, the intercellular distribution of DNA migration patterns was more homogeneous with increasing doses of CP.

Additional studies were conducted with mouse and/or rat liver parenchymal cells which were exposed to a variety of compounds, including diethylnitrosamine (DEN), ethylmethanesulphonate (EMS), and 2- and 4-acetylaminofluorene.

In Vivo Experiments

A series of experiments was conducted to evaluate the ability of ACR, DMBA and TCE to induce DNA damage in mice in four different tissues (brain, liver, spleen, blood). Male B6C3F1 mice were exposed acutely by gavage to 100 mg/kg ACR or DMBA, or to 1000 mg/kg TCE.

Four hours after treatment with ACR, cells from all four organs/tissues exhibited a significant increase in DNA migration, with liver cells showing the greatest percentage increase in response. By 24 hours after treatment, only blood leukocytes still exhibited an increased level of damage. None of the cells sampled four hours after treatment with DMBA exhibited an increase in DNA migration. However, at 24 hours after treatment, cells from all but brain exhibited a significant increase in DNA migration, with spleen cells showing the greatest response. Four hours after treatment with TCE, cells from all four tissues exhibited a significant increase in DNA migration, with spleen cells showing the greatest response.

These pilot studies demonstrated that the level of DNA damage induced by these chemicals was agent-, organ-, and sample time-dependent. They also showed the utility of the approach and the feasibility of detecting DNA damage in individual cells isolated from different organs of mice. However, the range of variation among cell samples from control mice (about 2-3 fold) was disappointing and led to an examination of factors involved in the processing of *in vivo* tissues.

Three factors were investigated. The collagenase treatment used to isolate single cells from brain and liver resulted in a significant increase (about 50%) in DNA migration. Mincing alone, without collagenase, was found to be sufficient for ensuring an adequate sample of single cells from every tissue tested. Secondly, it was found that the addition of calcium chelators, EDTA or EGTA, to the media solutions resulted in a very significant reduction in DNA migration in control cells. An adverse impact of blood in the lysing solution was corrected by adding DMSO.

Experiments with ACR subsequent to isolating these confounding factors demonstrated, under the modified sampling protocol, reproducible control data for each tissue between sample times, and reproducible data among animals at a specific dose.

Human Studies

One of the goals of this research was to be able to evaluate and compare data obtained on both animal and human populations. Thus, several pilot studies were conducted to examine the utility of the SCG assay in human biomarker investigations.

Blood from runners just completing a race, and from smoking vs nonsmoking populations was analyzed through the SCG assay. These preliminary pilot stud-

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ies provided equivocal results, with numerous possible explanations for these results.

In a more definitive study, the blood from patients undergoing chemotherapy at the Duke University Medical Center was analyzed. Patients with metastatic breast tumors were sampled before, during, and after intravenous administration of antineoplastic alkylating agents. The patients were exposed over a several-day period in a complex regimen to cyclophosphamide, cisplatin, and carmustine.

Results of the SCG assay are consistent across patients, with levels of DNA migration pre- and post-treatment being similar. DNA migration levels were elevated for samples taken during treatment. Thus, the data collected demonstrate the potential utility of the SCG assay in future human biomonitoring studies.

Hazardous Waste Site Studies

Feral rodents, *Ochrotomys nuttalli* (golden mouse), were live-trapped during May and June 1990 in this pilot study. Potentially exposed animals were taken from an area bordering the fenced North Carolina State University Superfund site. Predominant pollutants on the site include TCE, chloroform, carbon tetrachloride, various pesticides, laboratory solvents, and other chemicals. Control animals were trapped in nearby areas of similar ecology.

Blood, bone marrow, brain and liver tissues from 13 exposed and 13 control animals were examined via the SCG assay. Not surprisingly, the extent of interanimal variability was much greater than that observed normally for laboratory animals. The level of DNA damage, as measured by mean migration length, was increased in all four tissues of animals trapped near the Superfund hazardous waste site, but significantly only in brain (P=<0.05). However, a dispersion analysis revealed that the bone marrow cells from the mice living near or on the hazardous waste site exhibited a significantly increased dispersion coefficient over that calculated for the control mice (P=<0.05).

In any study, especially with wild-caught animals, the possible influence of animal health, food resources, etc., on the data collected must be recognized when analyzing those data. However, the results of this small pilot study indicate the potential usefulness of the SCG technique in evaluating DNA damage in free-living rodents.

Conclusion

Significant technical difficulties were encountered during the development and application of the SCG technique to *in vitro* and *in vivo* studies. The results of this research document that many of the problems have been surmounted and that the approach should be of considerable value to scientists attempting to evaluate animal and human populations for DNA damage induced by genotoxic agents acting as environmental pollutants.

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The complete report, entitled ""Evaluation of Exposure Markers," (Order No. PB91-144 675/AS; Cost: $23.00, subject to change) will be available only from:
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