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# Cell Cycle Disruption in Wild Rodent Populations as an Endpoint in Detecting Exposure and Effect

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Environmental pollutants manifest their detectable effects in natural fauna along a continuum ranging from acute lethality (i.e. rapid death of the organism) to those outwardly invisible. Characterizing and quantifying these "invisible", sublethal effects yields the most conservative estimates of risk to human and ecological health. Recently, scientists have begun to focus on developing and integrating bioassays which afford a finer resolution of these often "unobserved" effects (Bickham 1990, Dallas and Evans 1990, Murdoch and Hebert 1994, Shugart et al. 1992, Theodorakis and Shugart 1997). Bickham and Smolen (1994) propose the use of cytogenetic and molecular genetic techniques to identify heritable and somatic effects within individuals and populations to address both ecological and evolutionary concerns. In short, these techniques provide information for detecting short and long-term health effects and subsequent decisions addressing these concerns.

One such technique, flow cytometry (FCM), has been used at both the organismal and population level to detect sublethal chromosomal damage in exposed organisms (Bickham 1990, Bickham et al. 1992,1994, Custer et al. 1994, Dallas and Evans 1990, McBee and Bickham 1988). FCM is a sensitive indicator of cytogenetic damage and has been validated in both laboratory and field experiments (Bickham et al. 1992, 1994, McBee and Bickham 1988, Otto and Oldiges 1980, Otto et al. 1981a, 1981b). This technique measures chromosomal damage as elevated coefficients of variation (HPCV) in the DNA content of the cells within individuals and, subsequently, populations of individuals (Bickham 1990, Bickham et al. 1992, 1994, Custer et al. 1994, George et al. 1991, Lowcock et al. 1997, McBee and Bickham 1988). Another endpoint which can be measured using this technique is that of cell cycle proportions which estimates relative proportions of cells in the G0/G1, S, and G2+M phases of the cell cycle. Few environmental studies to date have attempted to measure and use these parameters (George et al. 1991). Analysis of the cell cycle has typically been used in clinical research to address concerns such as oncogenesis (e.g. aneuploidy) and cell cycle disruption following biological or chemical exposure (Gilbertz et al. 1998, Maier and Schawalder 1986, Watson et al. 1997).

Here we report the findings of a flow cytometric analysis of spleen tissue taken from two species of rodent inhabiting a Superfund site, Weldon Spring Military

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Training Area (WSMTA), in St. Charles county, eastern Missouri, an area contaminated with nitroaromatic compounds (e.g. TNT, DNT) and radionuclides. Using FCM, we investigated potential cell cycle alterations and clastogenic effects elicited by chronic exposure to low mean levels of nitroaromatic compounds and ionizing radiation in prairie voles *(Micotus ochrogaster)* and white-footed mice *(Peromyscus leucopus).* Both nitroaromatic compounds and radionuclides have been documented as mutagens (Donnelly et al. 1993, Lamb et al. 1995) and radionuclides have been documented as cell cycle disruptors as well (Gilbertz et al. 1998, Scholz et al. 1994). We were interested in investigating potential somatic DNA damage indicated by elevated HPCV's of the G0/G1 peak (2C DNA content) and/or cell cycle disruption in exposed populations of the two species.

## MATERIALS AND METHODS

Prairie voles and white-footed mice were collected in Sherman live traps from the Weldon Spring Military Training Area (WSMTA-6.7km$^2$), an Environmental Protection Agency Superfund site and from offsite reference areas. Prairie voles were collected from January-April 1993, from sites adjacent to a Department of Energy (DOE-0.9km$^2$) facility within the historical WSMTA contaminated with radionuclides and both radionuclides and nitroaromatic compounds (38°42'N 90°41'W). Though radioactive contamination at these sites has been documented, levels of radionuclide soil contamination have not been quantified. Prairie voles used as controls were trapped in uncontaminated areas within the WSMTA and areas offsite 0.8- l.2 miles southwest of the WSMTA. White-footed mice were collected from August-November 1994, from sites within the WSMTA contaminated with nitroaromatic compounds (38°42'N 90°44'W). These specimens were taken from areas with an average TNT soil contamination of 158.23mg/kg (Wickliffe 1997). White-footed mice used as controls were trapped in uncontaminated areas within the WSMTA and several offsite areas (38°35'N 90°32'W, 38°55'N 91°42'W, 38°31'N 90°34'W). Variation in exposure within in the WSMTA was a result of different ecological and habitat preferences between the two species in conjunction with the variable distribution of the contaminants (Wickliffe 1997). Rodents trapped in areas contaminated with radionuclides were grouped and designated RAD, those trapped in areas contaminated with both nitroaromatic compounds and radionuclides were grouped and designated TNT/RAD, those trapped in areas contaminated with nitroaromatic compounds were grouped and designated TNT, and those trapped in unpolluted areas were grouped and designated CONTROL.

Spleens were immediately harvested, placed in sterile, cryogenic vials, and stored in liquid nitrogen. Samples were subsequently shipped to Texas A&M University on dry ice and placed in an ultracold freezer (-80°C) prior to preparation and analysis.

Tissue blocks were prepared using the propidium iodide (PI) protocol of Vindelov and Christensen (1994) and assayed on a Coulter Epics Elite Flow Cytometer. Synthetic (DNACheck™-Coulter Corp., Hialeah, FL) and biological standards (isogenic *Gallus domesticus* erythrocytes, C57 Black *Mus domesticus* spleen) were prepared and used to optimize machine alignment and laboratory procedures prior to and intermittently during data collection. All samples for each experiment were analyzed consecutively on a single day to minimize technical variability. In addition, individuals belonging to specific treatment groups were unknown to the preparator and the machine operator. Ten thousand cells per individual were counted and the gated half-peak coefficient of variation (HPCV) in DNA content was calculated. Cell cycle data were generated by analyzing the ungated DNA histograms in the cell cycle analysis program Multicycle® (ver 3.11; Rabinovitch 1994). Histograms used in the analyses were quality checked prior to inclusion according to Shankey et al. (1993).

HPCV in DNA content and cell cycle proportion data between sites were statistically compared using non-parametric rank analyses (i.e. Wilcoxon 2-Sample Test or Kruskal-Wallis test) available in the SAS program (ver. 6.04) and deemed significantly different at an alpha level of 0.05. Only adult rodents were used in the analyses.

## RESULTS AND DISCUSSION

HPCV's in DNA content and cell cycle data were generated for 104 adult prairie voles. There were 17 voles in the CONTROL group, 24 voles in the RAD group, and 63 voles in the TNT/RAD group. No significant differences in HPCV, G1, or S-phase were observed among groups ($P>0.05$). Voles from the RAD and TNT/RAD groups however had a significantly lower percentage of cells in the G2+M phase of the cell cycle in comparison with voles from the CONTROL group ($P<0.0376$) (Table 1).

**Table 1.** Arithmetic mean percent cells in G2+M in prairie voles

| Group | %G2+M (Mean±SE) |
|---|---|
| CONTROL (n=17) | 8.83±0.91 |
| *RAD (n=24) | 6.99±0.65 |
| *TNT/RAD (n=63) | 6.59±0.37 |

*$P<0.04$

Thirty-seven white-footed mice were used to estimate HPCV in DNA content and cell cycle proportion data for CONTROL and TNT groups. There were 10 mice in the CONTROL group and 27 mice in the TNT group. No significant differences in HPCV, G1, or S-phase were observed among groups ($P>0.05$). However, mice from the TNT group had a significantly higher percentage of cells

in the G2+M phase in comparison with mice from the CONTROL group (P<0.03) (Table 2).

**Table 2.** Arithmetic mean percent cells in G2+M in white-footed mice

| Group | %G2+M (Mean±SE) |
|---|---|
| Control (n=10) | 6.09±0.66 |
| *TNT (n=27) | 9.21±0.73 |

*P<0.03

Two particularly interesting observations are apparent considering the history of the site and the cytometric results obtained. One rather striking observation is that following 30-40 generations of exposure to various levels of nitroaromatic and/or radionuclides, a discernible effect is still manifest in both species of rodent. Possibly, demographic discontinuities resulting from selective and/or stochastic processes affecting the distribution and concentration of the chemical composition, fauna, and flora have prevented an equilibrium from being achieved thus far. One result stemming from a selective response might be an ecological transition in which a subset of tolerant fauna predominate, but the diversity of mammalian fauna represented on WSMTA is very similar to that in offsite control areas suggesting no major ecological community shifts (Pitts et al. 1995). If a tolerant subset had colonized the area one might expect these individuals to exhibit no detectable effect. In contrast, a recurring influx of migrants may have colonized the contaminated areas from adjacent uncontaminated areas. If this were the case, biological perturbations such as cell cycle flux might be expected. Unfortunately, no evidence is available with which to address either of these hypotheses.

Secondly, the cell cycle anomalies themselves also present an intriguing point. Cell cycle disruption has been clearly demonstrated in numerous clinical studies where cytotoxic chemicals and ionizing radiation have been considered either a hazard or beneficial medical agent (Gilbertz et al. 1998, Maier and Schawalder 1986, Scholz et al. 1994, Watson et al. 1997). To our knowledge, this endpoint has not been investigated in the context of exposure to nitroaromatic compounds such as trinitrotoluene (TNT). Furthermore, cell cycle measurements have rarely been estimated in ecotoxicological surveys (George et al. 1991, Lamb et al. 1995). With respect to ecotoxicological research, considerable difficulty lies in defining the exact mechanism controlling or responsible for the observed effects. One reason for this stems, in part, from the complex chemical, ecological, and genetic history associated with varying spatial and temporal elements of exposure characterizing most field investigations. A concomitant lack of additional data complicates the interpretation as well. In addition, the vast majority of clinical observations indicating cell cycle anomalies consist of exposing immortal or mutant cell lines, which can be conveniently cultured in vitro, to fixed and/or cell cycle phase specific doses. These studies are invaluable in elucidating the nature of the damage, and the molecular mechanisms involved. In our estimation, such studies are difficult to extrapolate in simple terms to the results obtained here. For

example, studies on ionizing radiation have demonstrated increases in chromosomal aberrations and subsequent G2+M arrest (G2+M cell increase) prior to mitotic separation (Cistulli et al. 1996). However in using clonal or inbred strains, these studies avoid genomic variability and although the validity of these studies cannot be discounted, variation in the human population(s), with which risk assessment is assigned with protecting, is at least partially responsible for the spectrum of sensitivity observed following incidental exposure to hazardous substances. Thus, natural populations would seem to offer a more appropriate model for estimating risk though mechanisms governing observed effects are more difficult to discern.

As a result, a number of assumptions must be met in this study prior to extrapolating the potential mechanism controlling the observed effects from clinical studies. For example, one assumes the individuals inhabiting the contaminated areas are exposed and have similar ecological and genetic backgrounds. These two assumptions are likely met in this study. Additionally, one must assume exposure is relatively uniform and splenocytes (an asynchronous, rapidly cycling population) are effected in the same manner as clinical cell lines. Meeting these assumptions is more difficult. The decrease in the G2+M portion of the cell cycle observed in exposed prairie voles might be the result of fewer cells entering metaphase and possibly an indicator of apoptosis though no apoptotic subpeaking was observed. Clinical observations on cell cycle disruption following exposure to ionizing radiation typically indicate an increase in G2+M. Thus, there would seem to be different mechanisms responsible for the decrease in G2+M in exposed individuals in this study. The observed increase in G2+M in white-footed mice might be the result of post-synthetic arrest prior to repair and completion of telophase and cell fission. Though the data are difficult to explain without more analyses, the fundamental result is clear that individuals inhabiting the contaminated areas exhibit cellular alterations in comparison to individuals from nearby uncontaminated areas.

Finally, the value of biomarkers in pollution research has been well documented (Lyne et al. 1992, McCarthy and Shugart 1990, Shugart et al. 1992). The cell cycle data therefore provide an additional endpoint with which to monitor both individuals and populations. This endpoint allows researchers an opportunity to detect sublethal impacts including aneuploidy, polyploidy, phase distortion, and apoptosis. The challenge is to apply this technique in field experiments and compile the necessary information to reliably assess and diagnose the impacts environmental contaminants may pose. Finally, the potential impacts indicated in both species of small mammal should therefore inspire a more detailed analysis of the rodents inhabiting this Superfund site.

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