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Petrochemical-Related DNA Damage in Wild Rodents Detected by Flow Cytometry

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The need for quick, reliable, in situ tests of environmental mutagenicity is evidenced by increasing public concern about potential health effects of pollutants. Conventional tests of clastogenicity (one form of mutagenicity) usually involve treatment of laboratory test systems (e.g. mouse, cultured cell lines) with pure samples of suspect compounds followed by scoring numerous metaphase cells for chromosome aberrations (Hsu, 1982). There are at least two shortcomings of these test protocols. They are very time consumptive and are generally restricted to controlled laboratory situations which may not realistically indicate the effects of environmental pollution. The use of flow cytometry to study resident rodent species as bioindicators provides a system by which cytogenetic effects of environmental pollutants upon exposed organisms rapidly and accurately can be ascertained.

Flow cytometry has been an established method to measure relative DNA quantities per cell since the early 1970's. The utility of flow cytometry results from the speed, precision, and ease with which large numbers of cells can be analyzed for DNA content. Moreover, the assay is sensitive enough to distinquish among cells that differ by as little as 2-3% in DNA content (Deaven, 1982; Steen and Lindmo, 1979). The applicability of flow cytometric data to analysis of chemically induced chromosome damage has been suggested by several investigators (Deaven, 1982; Steen and Lindmo, 1979; Irons and Stillman, 1985). Because structural chromosomal aberrations cause an unequal distribution of DNA in daughter cells, growing cell populations should show an increased dispersion of cellular DNA content when impacted by clastogenic agents. Otto and Oldiges (1980) showed that the G_1 peak in somatic cells challenged with known mutagens had a typical broadened appearance and a higher coefficient of variation for mean DNA content than did untreated cells. Further studies (Otto et al., 1981) indicated that a dose-response relationship existed between the mutagen and the coefficient of variation of G_1 peaks.

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We (McBee et al., 1987; McBee, 1985) found that two species of wild rodents (Peromyscus leucopus and Sigmodon hispidus) living at a dump site polluted with a complex mixture of oil, grease, polychlorinated biphenols, hexachlorobenzene, zinc, manganese, cadmium, chromium, copper, and lead (Brown, 1980; Brown and Donnelly, 1982) had significantly higher frequencies of chromosomal aberrations than did animals from two unpolluted control sites. These data suggest that resident small mammals may be useful as in situ monitors of the presence and action of mutagenic pollutants in the environment. This study was conducted to determine if changes in patterns of DNA content indicative of the action of mutagens could be detected by flow cytometric analysis of tissues from these same animals.

MATERIALS AND METHODS

The principle study site was located at Brayton Field Firemen's Training School (FS), Texas A&M University, Brazos County, TX. The area has been used as a training ground for fire fighting exercises since 1961. Recently, it also has been used for training exercises in hazardous materials emergencies and oil drilling rig fire fighting. A summary of known contaminants and results of previous assays of extracts of soil, air, and water samples for mutagenicity are given elsewhere (Brown, 1980; Brown and Donnelly, 1982; Atlas et al., 1985). An unpolluted control site, similar in soil and vegetation patterns, was located approximately 1 km southwest of FS. Trapping procedures and animal selection and maintenance were as described by McBee et al. (1987). We analyzed flow histograms from 23 individuals of P. leucopus collected at FS and known to have increased frequences of chromosome aberrations. Histograms of DNA content in 13 P. leucopus collected from the control site with only background levels of chromosome aberrancy were also analyzed.

Spleen tissue was processed following procedures of Sherwood and Patton (1982). Tissues were minced with scissors and scalpel, washed twice in Hanks Balanced Salt Solution (BSS) and fixed in a 1:1 solution of cold (4°C) 95% ethanol and Hanks BSS. Cells were then filtered through 37 mu nylon monofilament mesh, and stored at 4°C for at least 24 h before staining with chromomycin A_3. Flow cytometry was performed with a Leitz MPV Flow Cytometer. The system was aligned and focused with 0.90 mu fluorescent microspheres and the gain controls used to adjust mean fluorescence intensity to channel 100. A standard prepared from the spleen cells of a male C57Bl/J6 Mus was used to adjust the gain so that the G_1 peak was at channel 100 and the coefficient of variation (CV) was at a minimal value (1.75-2.70).

All cell suspensions were number coded for double blind analysis. Five replicates of 10,000 cells were analyzed for each individual. Standard cells were run between every five to six individuals to readjust the gain and alignment of the system as

necessary. Fluorescence pulse height distributions were compiled and the mean fluorescence calculated by a Nuclear Data pulse height analyzer. The data were used to produce flow DNA histograms and CVs of each of the five runs per individual. Mean CVs for each individual in the two groups were calculated and statistically analyzed using the Mann- Whitney Rank Test. Following Otto and Oldiges (1980), mean CVs for each individual were also analyzed by calculation of a confidence range ($p < 0.05$) around the mean CV of all runs from control site animals. Values beyond the upper confidence limit of the control CVs were considered significantly increased and therefore, indicative of increased levels of DNA damage at the chromosomal level.

RESULTS AND DISCUSSION

Exemplary flow DNA histograms are shown in Fig 1. Coefficients of variation of mean DNA content and observed chromosome aberration levels for each animal are given in Table 1. Evidence of clastogenic activity was apparent in the broadened and flattened peaks and increased CVs of the G_1 peak in the animals from FS as compared to the animals from the control site and the C57Bl/J6 standard. FS animals showed a much broader range in CV values (2.12-8.70) than did control site animals (2.90-3.84).

The Mann-Whitney Rank Test indicated that CVs around mean DNA content of animals from the FS were significantly greater ($\alpha = 0.001$) than the CVs of animals from the control site.

Based on the calculated confidence range, any CV value exceeding the upper confidence limit (3.38) of the control mean was considered significantly increased. Thirteen (56.5%) of the 23 FS animals had CVs above 3.38, whereas only two (15.4%) of the control site values exceeded this value. Seven (30.4%) animals from FS had CVs that were below the lower confidence limit (3.05) whereas only three (23.0%) animals from the control site fell below the lower confidence limit. In all, 87% of the animals from FS had CVs that fell outside the control confidence range and only 38.4% of the control site individuals were not within upper and lower confidence limits.

Examination of flow DNA histograms also revealed two individuals from FS with subpopulations of aberrant cells. These were evident as either a subpeak of the G_1 peak (Fig 2) or as an additional small peak between G_1 and G_2 (Fig 1c). A possible explanation for aberrant subpeaks could be the occurrence of a stable subpopulation of aneuploid somatic cells, perhaps resulting from interruption of normal mitosis or a deletion in a single ancestral cell. Interestingly, the animal with the G_1 subpeak had one of the lowest levels of chromosome aberration at FS (Table 1).

Table 1. Coefficients of variation (CV) from FCM analysis of whole cell DNA content, number of aberrant cells (ABCELL) and mean number of lesions per cell (LES/CELL) from standard chromosome aberration analysis for Peromyscus leucopus collected at Firemen's Training School (FS) and the control site (CS). Dashes indicate animals for which chromosome aberration data were not obtained.

Specimen Number	CV 50,000 cells	ABCELL 50 cells	LES/CELL 50 cells	Locality
AK 9006	3.36	2	0.04	FS
AK 9008	5.80	6	0.14	FS
AK 9009	2.24	8	0.46	FS
AK 9010	3.50	4	0.08	FS
AK 9011	4.30	3	0.06	FS
AK 9012	2.94	5	0.12	FS
AK 9013	3.68	8	0.22	FS
AK 9015	2.62	-	-	FS
AK 9017	3.80	-	-	FS
AK 9018	3.52	-	-	FS
AK 9019	2.40	4	0.12	FS
AK 9020	2.40	4	0.04	FS
AK 9021	5.24	2	0.20	FS
AK 9030	3.12	2	0.60	FS
AK 9033	3.92	-	-	FS
AK 9034	2.12	-	-	FS
AK 9035	2.56	-	-	FS
AK 9037	8.70	-	-	FS
AK 9038	3.18	-	-	FS
AK 9041	4.46	-	-	FS
AK 9042	5.02	-	-	FS
AK 9043	5.42	-	-	FS
AK 9039	6.30	-	-	FS
AK 9022	2.92	1	0.10	CS
AK 9023	3.08	0	0	CS
AK 9031	2.90	2	0.04	CS
AK 9046	3.22	3	0.06	CS
AK 9047	3.05	4	0.08	CS
AK 9048	3.05	1	0.02	CS
AK 9049	3.20	3	0.06	CS
AK 9050	2.98	1	0.02	CS
AK 9051	3.38	2	0.04	CS
AK 9052	3.84	1	0.02	CS
AK 9053	3.76	2	0.04	CS
AK 9056	3.24	1	0.02	CS
AK 9057	3.42	-	-	CS

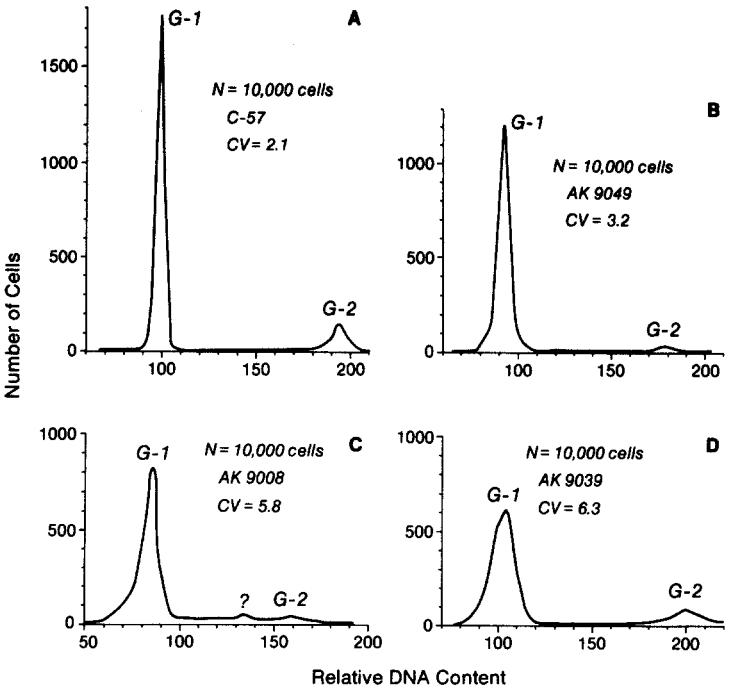

Figure 1. Flow DNA histograms of spleen, AK numbers refer to karyotype number, Wildlife Genetics Laboratory, Texas A&M University. The area under the G_1 peak includes 10,000 cells. Individual histograms are as follows: A. Mus C57Bl/J6; B,C,D all Peromyscus leucopus, B. from control site; C. from FS; D. from FS, ? indicates a small peak intermediate to G_1 and G_2 peaks.

The extra small peak observed in the S region of the flow DNA histogram of the other animal (Fig 1c) may indicate either a similar instance of an aneuploid subpopulation or the action of a DNA synthetic poison that acted to block synthesis at a particular stage causing an accumulation of cells which had only partially completed synthesis. These cells would have more DNA than the normal 2C amount characteristic of G_1 but less than the normal 4C amount characteristic of G_2.

The environmental toxicity, mutagenicity, and clastogenicity of petrochemical related pollutants is well documented (Lower et al., 1983; Pelroy et al., 1981; Lockard et al, 1982; Manabe et al., 1985; Matsuoka et al., 1982), but few studies actually deal with effects on natural populations of animals (Ma and Harris, 1985). This study demonstrates that flow cytometry may be a

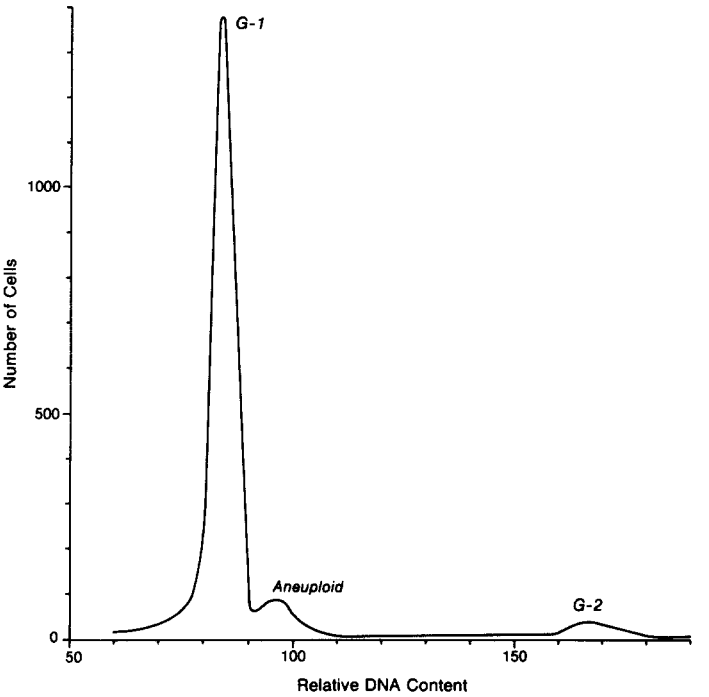

Figure 2. Flow DNA histogram of spleen from Peromyscus leucopus from FS. AK number refers to karyotype number, Wildlife Genetics Laboratory, Texas A&M University. The small subpeak at the base of the G_1 peak may represent a subpopulation of aneuploid cells. Area under the G_1 peak includes 10,000 cells, CV = 3.36.

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rapid, reliable way to assess the action of such environmental pollutants on the chromosomal complements of exposed organisms. Significant differences were found in amount of variation in whole cell DNA content in animals from FS and the control site. Furthermore specific subpopulations of abnormal cells also could be detected in some animals from FS. Although animals with atypical multiple peaks were observed with low frequency in this study, flow cytometric analysis may be a technique whereby aneuploidy can be readily detected. These data encourage intensified evaluation of the use of flow cytometric analysis of resident rodent species as a rapid and accurate in situ screen for the presence and action of environmental mutagens.

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