ELSEVIER

Mutation Research 441 (1999) 163–170

Genetic Toxicology and Environmental Mutagenesis

www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Induction of micronuclei in V79 cells by fractions of roofing asphalt fume condensate

H.-W. Qian [a,b], W.-Z. Whong [a], L. Olsen [c], J. Nath [b], T. Ong [a,b,*]

[a] *National Institute for Occupational Safety and Health, ALOSH, Room 3014, 1095 Willowdale Road, Morgantown, WV 26505, USA*
[b] *West Virginia University, Morgantown, WV, USA*
[c] *National Institute for Occupational Safety and Health, 5555 Ridge Avenue, Cincinnati, OH, USA*

Received 25 June 1998; received in revised form 22 February 1999; accepted 24 February 1999

Abstract

More than 50,000 workers in the United States are exposed to roofing asphalt fumes that may pose genotoxic and potential carcinogenic hazards. The Type III roofing asphalt is most frequently used in roof-application. Results of our previous studies showed that fume condensates of Type III roofing asphalts induced micronuclei (MN) in vitro in cultured V79 cells and DNA adduct formation in vivo in rat lung cells. In this study, the genotoxicity of whole fume condensates (WFC) of Type III roofing asphalt and its five chemical fractions (A, B, C, D and E) was determined by the micronucleus assay using V79 cells. Linear regressions were determined for the dose response of MN frequencies and percent of binucleated and multinucleated cells (MTC) following the treatment. Results showed that the numbers of micronucleated cells in cultures treated with Type III roofing asphalt WFC and its fractions B, C, D and E were significantly higher than that in the control culture, and that the slopes of the linear regression line for fractions B and C were greater than those for the WFC and fractions D and E. A clear dose response of binucleated cells was also induced by the WFC and fractions B and C. These findings indicate that: (1) WFC and all fractions, except fraction A, induced MN formation in cultured V79 cells; (2) fractions B and C possess the highest genotoxic activity; (3) the roofing asphalt WFC contains chemicals or chemical classes that induce not only chromosomal aberrations but also binucleation in V79 cells.

Keywords: Roofing asphalt fume; Fraction; Micronucleus; Binucleated cell; V79 cell; In vitro

1. Introduction

Asphalt is made from crude petroleum by distillation. The composition of asphalt is quite complex and depends on the source of oil, distillation process, melting temperature, etc. [1]. Asphalt fume, generated at high temperatures from raw asphalt, contains a complex mixture of organic compounds including aliphatic (~80%) and aromatic (~20%) compounds [2]. Efforts have been made to identify the chemical classes in roofing asphalt fume mixture that may be responsible for possible adverse health effects. Based on polarity, the whole fume condensate (WFC) was separated by high performance liquid

* Corresponding author. Tel.: +1-304-285-6233 ext. 1; Fax: +1-304-285-6194

1383-5718/99/$ - see front matter
PII: S1383-5718(99)00045-5

Table 1
Major chemical classes identified by GC/MS in the different fractions of whole fume condensates[a]

Fraction	Composition
A	C9 to C35 alkanes and monocyclic alkanes, alkylated benzenes, alkylated naphthalenes, benzothiophenes, biphenyls, fluorenes, indanes, indenes
B	Alkylated benzo- and dibenzo-thiophenes, alkylated benzo-naphthothiophenes, alkylated anthracenes and phenanthrenes, benzo- and dibenzo-furans, C6 to C26 olefins, fluoranones, pyrene and fluoranthrenes
C	Alkylated phenylethanones; C2 to C11 alkylated dihydrofuranones, dihydrofuranones; alkylated cyclo ketones; isobenzo furanones; hydroxy benzenethiols; tricarbocyclic fused ring thiophenes; chrysenes
D	Alkylated phenols, alkylated ketones and acids, carbazoles, furanones
E	C6 to C22 alkylated ketones and acids, alkylated naphthols and phenols, benzoic acids

[a] Sivak et al. (1989) [4].

chromatography (HPLC) into five fractions, designated A, B, C, D and E [3]. The carcinogenic potential of these fractions has been tested in mice. Following chronically local painting, fractions B and C were found to be tumorigenic in mouse skin. The other three fractions produced neither initiation nor promotion in skin tumorigenesis [4,5]. Different mutagenicity results in bacteria by the fractions of roofing asphalt WFC were also found in the Salmonella microsomal assay [6]. In the presence of Arochlor-induced hamster liver S9 mix, Fractions B and C have been shown to be mutagenic to *Salmonella typhimurium* TA98; also, fractions A and D produced a weak mutagenic response in TA98. No mutagenicity in *Salmonella* was found for fraction E. However, no additional information regarding the genotoxicity of these fractions of asphalt WFC is available.

The in vitro micronucleus (MN) assay, which detects chromosomal breakage and/or aneuploidy, is commonly used in the assessment of the genotoxicity of chemicals to mammalian cells [7–10]. Recently, we found that roofing asphalt WFC induced MN formation in cultured Chinese hamster lung fibroblast (V79 cells) primarily by damaging the spindle, and to a lesser extent, by breaking chromosomes [11]. Further studies were carried out to determine whether (1) any of the five fractions of roofing asphalt WFC can induce MN formation in V79 cells, (2) there is any correlation between carcinogenicity and genotoxicity of fractions, and (3) any of the fractions can induce bi- and multinucleated cells (BNC and MTC). Binucleation and multinucleation are indicators of abnormal cell division due to blocking of cytokinesis [12]. This abnormal cell division would result in genetic imbalance in the cells that may also be involved in carcinogenesis [13–16].

2. Materials and methods

2.1. The source of fume condensate and its fractions

Type III roofing asphalt fume condensates and their fractions were produced by Sivak et al. [4]. The methods for the fume condensate preparation and its HPLC fractionation have been described by Belinky et al. [3] and Sivak et al. [4]. Briefly, roofing asphalt was broken in small pieces and placed in a 12-l round bottom flask which was heated to 316 ± 10°C on an electric heating mantle. This temperature is similar to the high overheat temperature reach in

Notes to Table 2:
[a] Relative colony forming efficiency.
[b] 3000 cells were scored.
[c] Solvent control (0.25% DMSO).
[d] Trend test $P < 0.01$.
[e] *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (positive control).
[*] Comparred to control, $P < 0.05$.
[**] $P < 0.01$ (χ^2 test).

asphalt roofing kettles in the field. The fumes generated from asphalt were cryotrapped in glass impingers filled with organic solvents (50/50 mixture of cyclohexane/acetone), the glass impingers were immersed in a slurry of dry ice/isopropyl alcohol. Collected materials from all impingers and tubing

Table 2
Frequency of micronucleated cells (MNC) in V79 culture treated with WFC and its fractions

Chemical	Concentration (g/ml)	CFE[a] (%)	Total number of MNC[b]	Frequency of MNC (‰) (mean, SE)
Whole fume condensate				
	0[c]	100.0	11	3.7, 0.3[d]
	31.3	80.6	18	6.0, 1.5
	62.5	53.4	28	9.3, 0.3**
	125.0	24.8	62	20.7, 1.5**
	187.5	6.8	65	21.7, 3.0**
	1 (MNNG)[e]	52.9	162	54.0, 2.3**
Fraction A				
	0[c]	100.0	18	6.0, 1.0
	62.5	90.7	13	4.3, 0.3
	125.0	80.9	20	6.7, 1.3
	187.5	54.9	22	7.3, 0.7
	250.0	23.3	23	7.7, 1.5
	1 (MNNG)[e]	48.8	138	46.0, 0.6**
Fraction B				
	0[c]	100.0	11	3.7, 0.3[d]
	7.8	90.8	23	7.7, 0.3*
	15.6	83.0	24	8.0, 1.5*
	31.3	67.5	46	15.3, 1.3**
	62.5	7.8	118	39.3, 1.2**
	1 (MNNG)[e]	52.9	162	54.0, 2.4**
Fraction C				
	0[c]	100.0	14	4.7, 2.2[d]
	7.8	104.7	13	4.3, 0.9
	15.6	94.5	32	10.7, 1.2**
	31.3	57.9	76	25.3, 2.8**
	62.5	13.6	106	35.3, 0.7**
	1 (MNNG)[e]	47.3	185	61.7, 3.7**
Fraction D				
	0[c]	100.0	10	3.3, 1.2[d]
	15.6	87.1	16	5.3, 0.7
	31.3	67.1	12	4.0, 0.6
	62.5	45.6	29	9.7, 1.2**
	125.0	9.6	67	22.3, 1.5**
	1 (MNNG)[e]	39.8	185	61.7, 4.9**
Fraction E				
	0[c]	100.0	13	4.3, 0.9[d]
	7.8	86.6	17	5.7, 0.9
	15.6	65.2	15	5.0, 0.6
	31.3	45.1	36	12.0, 1.5**
	62.5	1.6	50	16.7, 2.7**
	1 (MNNG)[e]	33.6	149	49.7, 1.5**

were combined and were separated into a water phase and an organic solvent phase using a 2-l separatory funnel. Water was removed using a vacuum oven at 690 mm Hg and 45–55°C, and the organic solvents was removed using a rotary evaporator at a reduced pressure with a water aspirator and the same temperature. After removal of the solvents (water and cyclohexane/acetone), the remaining materials were dissolved in a 50/50 (v/v) cyclohexane/acetone solution so that a 50% w/v (g/ml) solution of asphalt fumes was obtained. Before HPLC fractionation, the asphalt fume solutions were solvent exchanged with a mixture of hexane and methyl t-butyl ether using the rotary evaporation procedure used previously. These solutions were then filtered (0.45 mm) and separated on an aminopropyl-bonded phase column (Water NH_2 Prep HPLC column) using a Waters Autoprep 500, a liquid chromatographic system equipped with two Waters UV detectors. Table 1 shows the major chemical classes present in the different fractions of the WFC identified by GC/MS [4].

2.2. Cell line and culture

The Chinese hamster lung fibroblast cell line (V79) was kindly supplied by Dr. C.C. Chang (Michigan State University, East Lansing, MI). Cells were grown exponentially in minimum essential medium (MEM; Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), 1 mM L-glutamine (Sigma), 100 units penicillin/ml, and 100 mg streptomycin/ml (Sigma). Cultures were maintained in 75-cm^2 Falcon tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. They were subcultured twice a week using 0.25% trypsin solution (Sigma) in phosphate buffered saline (PBS).

2.3. Micronucleus assay

Exponentially growing cells were subcultured at a concentration of 5×10^5 cells/60 mm dish for each treatment. Cells in each dish were incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 h. The roofing asphalt WFC and each fraction were dissolved in DMSO individually and added to each culture, respectively. Four different concentrations which gave approximately 10 to 100% relative colony forming efficiency were tested for each sample. DMSO and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were used as solvent and positive controls, respectively. Cells were treated for 24 h and were then washed twice with PBS and fresh medium was placed in each dish. After an additional 24-h post-treatment incubation, cells were detached by trypsinization, collected by centrifugation, and resuspended in culture medium at a density of 1×10^6 cells/ml. An aliquot of the cell suspension (60–80 ml) was loaded into a chamber and the cells were pelleted onto slides using Shandon Cytospin II at 600 rpm for 7 min. The slides were left to air dry, fixed with absolute methanol, and stained with Diff-Quik stain (Fisher, Pittsburgh, PA). The micronucleated cells (MNC) were scored using criteria described by [17]. The results were expressed as the mean number of MNC per 1000 cells. A cell with two (approximately same size) nuclei is classified as a BNC and with three or more (approximately same size) nuclei is classified as a multinucleated cell (MTC). A total of 1000 cells were counted to determine the percentage of BNC and MTC. All experiments were repeated at least once.

2.4. Statistical analysis

The χ^2 test was used for a comparison of mean value. The dose response of cultured V79 cells to the respective chemical treatment was analyzed by the trend test. Correlation coefficients were calculated between concentration and net MN frequency and tested by the *t*-test. The equation of the linear regression line for different samples was estimated. The comparisons of relative genotoxic potency among WFC and its fractions were performed by the slope of linear regression line using the covariance test. All statistical tests were two-tailed analysis and critical values were determined using a 0.05 and 0.01 probability of type I error [18].

3. Results

Table 2 shows that the treatment of V79 cells with WFC and its fractions B, C, D and E caused a significant increase in the frequency of MNC; how-

Table 3
Linear relationship between the net increases in frequency of MNC induced by WFC and its fractions in V79 cells in vitro

Roofing asphalt fume	Linear regression equation ($Y = a + bX$)[a]	Correlation coefficient (γ)	t-Test for γ	Predictive concentration range (μg/ml)
Neat condensate	$Y = -0.31 + 0.11X$	0.94	$P < 0.05$	31.3–187.5
Fraction B	$Y = -3.81 + 0.60X$	0.98	$P < 0.01$	7.8–62.5
Fraction C	$Y = -1.71 + 0.55X$	0.96	$P < 0.05$	7.8–62.5
Fraction D	$Y = -2.79 + 0.17X$	0.97	$P < 0.05$	15.6–125.0
Fraction E	$Y = -0.80 + 0.22X$	0.96	$P < 0.05$	7.8–62.5

[a] X, the concentration used in the experiment.
Y, predictive net frequency of mononucleate cells with MN (‰).
a, Intercept.
b, The slope.

ever, fraction A did not cause a significant increase in the MNC frequency at concentrations up to 250 mg/ml. The lowest effective concentration was different for each sample, and was in order of fractions B (7.8 mg/ml) < C (15.6 mg/ml) < E (31.3 mg/ml) < D and WFC (62.5 mg/ml). A lower effective concentration represents higher genotoxicity to V79 cells.

The dose response of the net increase in MNC frequency induced by various concentrations of WFC and its fractions was estimated using linear regression equations (Table 3). Within the test concentra-

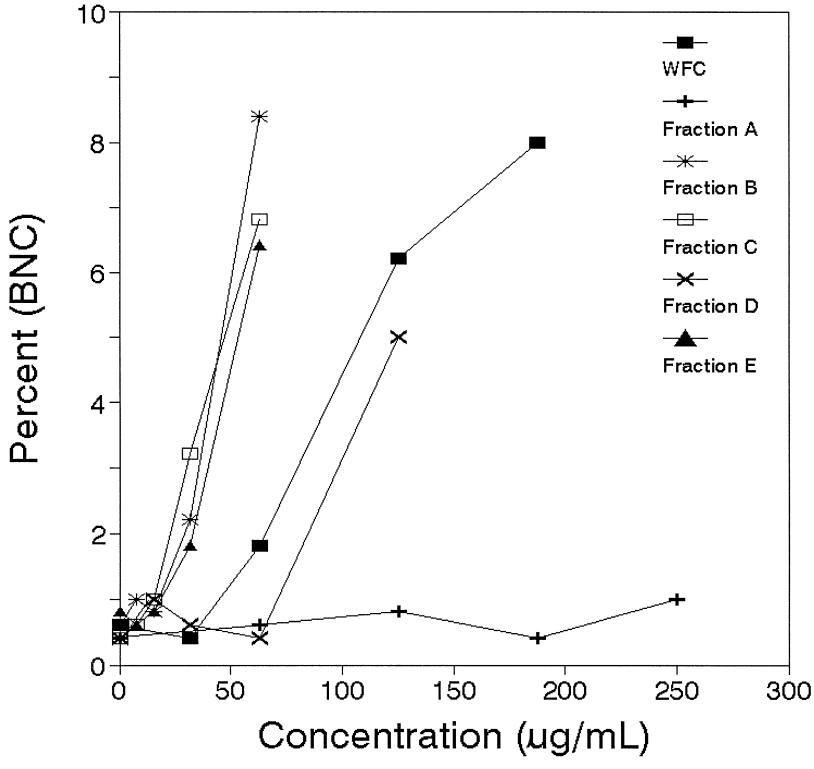

Fig. 1. The percent of BNC in V79 cells following treatment with WFC and its fractions (A–E).

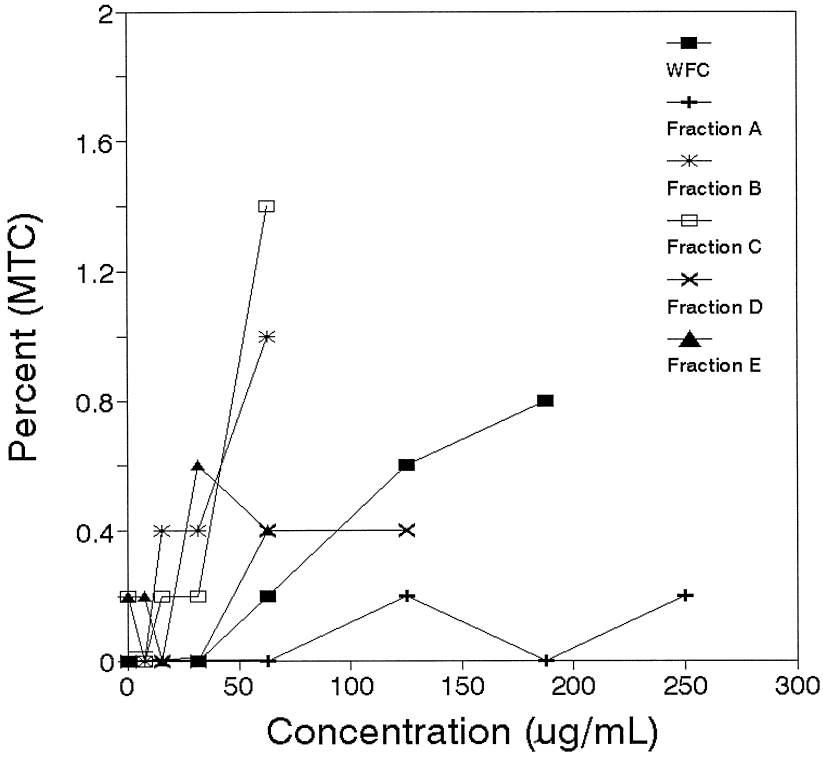

Fig. 2. The percent of multinucleated cells (MTC) in V79 cells following treatment with WFC and its fractions (A–E).

tion range, a significant correlation (γ) was noted for WFC (0.94), fraction B (0.98), C (0.96), D (0.97), and E (0.96). The slopes were compared between various samples using the covariance test. The slopes were much greater in fractions B and C compared to those in WFC and fractions D and E. However, there were no significant differences between the slopes (b; as described in Table 3) of fractions B (0.60) and C (0.55). Similarly, the slopes of fractions D (0.17), E (0.22) and WFC (0.11) were not significantly different from each other.

Fig. 1 shows that there was an increasing trend in the proportions of BNC for all test samples except for fraction A. Clear dose responses were found in WFC and fractions B and C (trend test, $P < 0.05$). Significant BNC formation was observed only at the highest concentrations, 125 mg/ml for fraction D and 62.5 mg/ml for fraction E (χ^2 test, $P < 0.05$). In addition, an increasing trend in the proportion of MTC seemed to exist for WFC and fractions B, C and D (Fig. 2), but did not reach a significant level at any of the concentrations used (Trend test, $P > 0.05$). The lower percentage of MTC may be due to a short period of post-treatment incubation.

4. Discussion

The results from this study indicate that WFC of Type III roofing asphalt and its fractions, except fraction A, induced MN formation in V79 cells. The relative potency of the WFC and its fractions to cause cytogenetic damage was compared using the slope of the linear regression line which represents net increase in MN frequency per unit of test chemical [18]. The higher the value of slope, the steeper the linear regression line, which indicates a more genotoxic effects of the test chemical. Since the slope in fractions B and C is much steeper than that in WFC and fractions D and E, these fractions (B and C) had the highest genotoxic activity. The geno-

toxicity of fractions D and E in V79 cells appeared to be similar to those of WFC. Results of our previous study showed that WFC induced MN in V79 cells mainly by damaging the spindle apparatus [11]. It is not known whether fractions of WFC induced MN by the same mechanism.

Induction of DNA adducts in vivo in rat lung cells has been found following the intratracheal instillation of Type I and Type III roofing asphalt WFC generated at 316°C [19]. With the Ames Salmonella microsomal assay, similar genotoxic results have also been reported for the roofing asphalt WFC [6]. Fractions B and C are mutagenic to *S. typhimurium*. However, fractions A and D are weakly mutagenic and fraction E is non-mutagenic. Both fractions B and C have been shown to be tumorigenic in mouse skin painting studies. As shown in Table 1, Fraction B mainly contains alkylated benzo- and dibenzothiophenes, and alkylated benzo-naphthothiophenes while fraction C mainly contains alkylated phenylethanones and dihydrofuranones [4]. These chemicals may be responsible for the genotoxicity and carcinogenicity of fractions B and C. Disagreements of different assay systems on the genotoxic potential of fractions A, D and E of Type III roofing asphalt WFC may be the result of system-sensitivities to a particular fraction tested. Endpoints of genotoxic assays may reflect a DNA/chromosomal lesion or other genetic change caused by a specific chemical or chemical group [20–23]. Fractions A, D and E tested are chemically grouped complex which may represent a case where not all genotoxic assays are common to their chemical genotoxicants.

The formation of BNC is an indication of the effect of chemicals on cell division, specifically cytokinesis [12]. Microtubes and microfilaments are involved in the cell division process. The microtube asters may initiate the cleavage-furrow formation and determine the position of contractile-ring formation. The contractile ring formed from microfilaments then further completes the cleavage [24]. Damage to these cytoskeleton fibers may block cytokinesis and lead to the formation of BNC [25]. Results of our studies show that WFC of type III roofing asphalt and its fractions, except fraction A, induced BNC in V79 cells. It seems, therefore, that roofing asphalt fumes contain chemicals or chemical classes which can induce MN and inhibit cytokinesis by damaging spindle fibers [11] and microfilaments, respectively.

In conclusion, this study suggests a potential risk associated with exposure to certain chemical components of roofing asphalt WFC. The correlation of the positive findings of the in vitro MN assay and the in vivo animal carcinogenic assay seem to indicate that fractions B and C in asphalt WFC may contain carcinogenic subfractions. Due to the chemical complexity of fractions B and C, it would be worth isolating the chemical subfractions and further detailing their genotoxic and carcinogenic potential.

Acknowledgements

The authors would like to thank Mr. John Stewart and Dr. Bi Song for their laboratory assistance. This work was performed at the National Institute for Occupational Safety and Health. Published with approval of the director of Agriculture, Forestry and Consumer Science Experiment Station as Scientific Paper Number: 2639.

References

[1] V.P. Puzinauskas, L.W. Colbett, Difference between petroleum asphalt, coal-tar pitch, and road tar, College Park, MD, Asphalt Institute Research Report No. 78-1, unpublished report, 1978.

[2] P.S. Thayer, J.C. Harris, K.T. Menzies, Integrated chemical and biological analysis of asphalt and pitch fumes, in: M.D. Waters, S.S. Sandhu, J. Lewtas, L. Claxton, N. Chernoff, P. Nesnow (Eds.), Short Term Bioassays in the Analysis of Complex Environmental Mixtures III, Plenum, New York, 1983, pp. 351–366.

[3] B.R. Belinky, C.V. Cooper, R.W. Niemeier, Fractionation and analysis of asphalt fumes for carcinogenicity testing, Proceedings of the fourth NCI/EPA/NIOSH collaborative workshop: Progress on joint environmental and occupational cancer studies, NIH Publication No. 88-2960, 1988, pp. 119–128.

[4] A. Sivak, K. Menzies, K. Beltis, J. Worthington, A. Ross, R. Latta, Assessment of the cocarcinogenic/promoting activity of asphalt fumes, Cincinnati, OH, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Institute for Occupational Safety and Health, Division of Biomedical and Behavioral Science, Contract No. 200-83-2612, 1989.

[5] A. Sivak, R. Niemeier, D. Lynch, K. Beltis, S. Simon, R. Salomon, R. Latta, B. Belinky, K. Menzies, A. Lunsford, C. Cooper, A. Ross, R. Bruner, Skin carcinogenicity of con-

densed asphalt roofing fumes and their fractions following dermal application to mice, Cancer Lett. 117 (1997) 113–123.
[6] NTP, NTP Results Report, Results and status information on all NTP chemicals produced from NTP Chemtrack System, Washington, DC, National Toxicological Program, 1990.
[7] H.J. Evans, Historical perspectives on the development of the in vitro micronucleus test: a personal view, Mutat. Res. 392 (1997) 5–10.
[8] D. Marzin, The position of the in vitro micronucleus test with the battery of screening for genotoxic potential determination and regulatory guidelines, Mutat. Res. 392 (1997) 175–181.
[9] J.D. Tucker, R.J. Preston, Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges, and cancer risk assessment, Mutat. Res. 365 (1996) 147–159.
[10] M. Kirsch-Volders, A. Elhajouji, E. Cundari, P.V. Hummelen, The in vitro micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breaking, chromosome loss and non-disjunction, Mutat. Res. 392 (1997) 19–30.
[11] H.W. Qian, T. Ong, W.Z. Whong, Induction of micronuclei in cultured mammalian cells by fume condensates of roofing asphalt, Am. J. Ind. Med. 29 (1996) 554–559.
[12] V. Rodilla, Origin and evolution of binucleated cells and binucleated cells with micronuclei in cisplatin-treated CHO culture, Mutat. Res. 300 (1993) 281–291.
[13] K. Pelin, P. Kivipensas, K. Linnainmaa, Effects of asbestos and man-made vitreous fibers on cell division in cultured human mesothelia cells in comparison to rodent cells, Environ. Mol. Mutagen. 25 (1995) 118–125.
[14] L. Sargent, Y. Dragan, Y.H. Xu, G. Sattler, J. Wiley, H.C. Pitot, Karyotypic changes in a multistage model of chemical hepatocarcinogenesis in the rat, Cancer Res. 56 (1996) 2985–2991.
[15] M.W. Beckmann, D. Niederacher, H.J. Schnurch, B.A. Gusterson, H.G. Bender, Multistep carcinogenesis of breast cancer and tumour heterogeneity, J. Mol. Med. 75 (1997) 429–439.
[16] S.T. Ong, M.M. Le Beau, Chromosomal abnormalities and molecular genetics of non-Hodgkins lymphoma, Semin. Oncol. 25 (1998) 447–460.
[17] P.I. Countryman, J.A. Heddle, The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes, Mutat. Res. 41 (1976) 321–332.
[18] S. Dowdy, S. Wearden (Eds.), Statistics for Research, Wiley, New York, 1991.
[19] H.W. Qian, T. Ong, J. Nath, W.Z. Whong, Induction of DNA adducts in vivo in rat lung cells by fume condensates of roofing asphalt, Teratog. Carcinog. Mutagen. 18 (1998) 131–140.
[20] B.L. Pool, Short-term tests as a tool in the identification of combinations and of combination effects in carcinogenesis, in: D. Schmähl (Ed.), Combination Effects in Chemical Carcinogenesis, VCH Verlagsgesellschaft, Weinheim, Germany, 1988, pp. 45–64.
[21] F.K. Ennever, H.S. Rosenkranz, Prediction of carcinogenesis potency by short-term genotoxicity test, Mutagenesis 2 (1987) 39–44.
[22] P.G. Shields, Inherited factors and environmental exposures in cancer risk, J. Occup. Med. 35 (1993) 34–41.
[23] M.D. Waters, H.F. Stack, M.A. Jackson, H.E. Brockman, Interpretation of short-term test data: implications for assessment of chemopreventive activity, IARC, Scientific Publication No. 139, IARC, Lyon, 1996, pp. 313–332.
[24] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, J.D. Watson (Eds.), Molecular Biology of The cell, Garland, New York, 1994, pp. 934–938.
[25] S.K. Aggarwal, M.W. Whitehouse, C. Ramachandrai, Ultrastructural effects of cisplatin, in: A.W. Prestayko, S.T. Crooke, S.K. Carter (Eds.), Cisplatin—Current Status and New Developments, Academic Press, New York, 1980, pp. 79–111.