Diesel exhaust particulate material expression of in vitro genotoxic activities when dispersed into a phospholipid component of lung surfactant

Shi X C, Keane M J, Ong T M, Harrison, J C, Slaven J E, Bugarski A D, Gautam M, Wallace W E.

US Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, WV, USA

Abstract. Bacterial mutagenicity and mammalian cell chromosomal and DNA damage in vitro assays were performed on a diesel exhaust particulate material (DPM) standard in two preparations: as an organic solvent extract, and as an aqueous dispersion in a simulated pulmonary surfactant. U.S. National Institute for Standards and Technology DPM SRM 2975 expressed mutagenic activity in the *Salmonella* reversion assay, and for in vitro genotoxicity to mammalian cells as micronucleus induction and as DNA damage in both preparations: as an acetone extract of the DPM mixed into dimethylsulfoxide, and as a mixture of whole DPM in a dispersion of dipalmitoyl phosphatidyl choline. Dispersion in surfactant was used to model the conditioning of DPM depositing on the deep respiratory airways of the lung. DPM solid residue after acetone extraction was inactive when assayed as a surfactant dispersion in the micronucleus induction assay, as was surfactant dispersion of a respirable particulate carbon black. In general, a given mass of the DPM in surfactant dispersion expressed greater activity than the solvent extract of an equal mass of DPM.

1. Introduction

Some organic solvent extracts of filter-collected diesel exhaust particulate material (DPM) express in vitro genotoxic activities to bactetria or mammalian cells [1,2]. These activities vary with engine design and operating conditions, fuels, and other engineering factors [3]. We have reported that some filter-collected DPM also can express bacterial mutagenicity and in vitro mammalian cell chromosomal or DNA damage activities when the material is mixed into an aqueous dispersion of simulated lung surfactant, e.g., aqueous dispersion of dipalmitoyl phosphatidyl choline (DPPC) [4,5]. Genotoxic activity is not expressed by the DPPC extract [6]; instead it resides with the non-dissolved but surfactant-dispersed solid ultrafine particles.

In the study reported here, a U.S. National Institute for Standards and Technology standard DPM reference material, SRM 2975, was assayed as both a solvent extract and as a surfactant dispersion in three in vitro assays: bacterial mutagenicity as Salmonella reversion to histidine independence, using the YG1024 and YG1029 tester strains with and without microsomal enzyme S9 activation; mammalian cell clastogenic activity as micronucleus induction in Chinese hamster fibroblast-derived V79 cells; and DNA damage as single- or double-strand breaks in V79 cells. SRM 2975 was prepared as an acetone extract mixed into dimethylsulfoxide; or as whole DPM mixed into an aqueous dispersion of DPPC. To investigate the role of particles *per se*, that is, particles bearing no solvent-

extractable genotoxicants, micronucleus assays also were performed on DPPC surfactant dispersion of the residue of previously acetone-extracted SRM 2975, and on a respirable particulate carbon black.

2. Materials and methods

2.1. Diesel particle material (DPM)
Standard Reference Material, SRM 2975, a forklift-generated diesel exhaust particulate material from the U.S. National Institute of Standards and Technology, was used for the study.

2.2. Treatment of samples

2.2.1. *Solvent extraction* For preparation of DPM- solvent extract, 500 mg of the SRM 2975 DPM and 100ml of acetone were sonicated together in water bath for 2 h. Then the liquid was evaporated under N2 to reduce the volume to <40ml, and then centrifuged at 3000xg for 30 min. The supernatant was removed and syringe-filtered. The filtrate was evaporated to dryness under N2. This acetone extraction procedure yielded extract material equal to 4% of the mass of the original SRM2975 DPM when performed for samples for mutagenicity testing; 5.6% for samples for micronucleus and DNA damage assays, and 8 % for samples for micronucleus comparisons of DPPC-dispersed carbon black with extracted or dispersed DPM. This evaporated solvent extract residue was mixed into dimethylsulfoxide (DMSO) at 36 mg DPM-extract per ml DMSO solution as a stock solution. Samples were diluted serially with complete Eagle's minimal essential medium (MEM, Gibco) to desired concentrations for in vitro assay.

2.2.2. *Surfactant Dispersion* For the preparation of surfactant dispersion, 2.5mg DPPC (Calbiochem, La Jolla, CA) per ml was mixed with 0.85% physiological sterile saline (PSS) and sonicated under N2 at 40 W (Heat system-ultrasonic, Plainview, NY) for 10 min. The probe was wiped with 95% ethanol and was covered with parafilm during sonication. Two mg DPM per ml DPPC stock dispersion was made after agitating the sample in a water bath for 60 min at 37ºC. The sample was diluted serially with complete Eagle's MEM medium to desired concentrations.

For surfactant dispersion of acetone-extracted SRM 2975 residue or of carbon black, 2mg per ml DPPC dispersions were prepared and diluted serially with complete medium to required concentrations.

2.2.3. Ames Salmonella Assay Mutagenic activity was analyzed by the pre-incubation variant of the Ames Salmonella microsuspension assay system [7]. Two bacterial test strains, YG1024 and YG1029, were used to identify frame-shift and base- pair substitution mutations. A 10% concentration of rat liver microsomal fraction S9 was used for metabolic activation for half of the samples. Co-incubation of bacteria, test sample, and S9 was performed by a rotary shaker at 37ºC for 30 min before plating. Duplicate plates were made for each concentration of test sample and the control. Three readings of revertant colonies per plate were scored by an automatic colony counter (Accu Count 1000, Biologics Inc., Gainville, VA) at 48 h for YG1029 and at 72 h for YG1024 after incubation. The average number of revertant colonies and standard deviation per concentration were calculated. A sample was considered to be positive for mutagenic activity if the number of revertant colonies per plate was twice as high as that of the DMSO solvent control or DPPC surfactant control in at least one concentration and if there was a clear concentration-response relationship.

2.1.4 Micronucleus (MN) Assay Micronucleated cells (MNC) were scored according to specific criteria [8,9]. Micronuclei located within the cytoplasm but separated from the main nuclei and with diameter between one-twentieth and one-fifth that of the nucleus were scored as a MN. The frequency of micronucleated cell (MNC) expressed as the number of MNC per 1000 cells was based on 6000 cells scored for each treatment. Chinese hamster lung fibroblasts (V79 cells) at a concentration of 1

×106 were cultured in a 25ml flask containing 5 ml of Eagle's minimal essential medium (MEM, Gibco) supplemented with 10% inactivated fetal bovine serum and 2% penicillin-streptomycin in a humidified incubator at 37ºC in an atmosphere of 5% CO2 for 24 h. Cells were treated for 24 h in 5ml of fresh complete MEM medium containing the test sample which had been prepared as a solution in 0.5% DMSO or dispersion in DPPC. Cytotoxicity of V79 cells after treatment was determined by cell density measurement and viability was determined by using the trypan blue exclusion assay. Cells were rinsed with PBS and re-incubated in 5ml fresh complete MEM medium for an additional 24 h. Cells were harvested by trypsinization, collected by centrifugation, and re-suspended in medium at about 4-5× 105/ml. Slides were prepared by a cytocentrifuge (Shandon Cytospin 3, Shandon Inc., Pittsburgh, PA). An aliquot of 75µl of the cell suspension was added into a chamber and the cells were pelleted onto the slide at 500 rpm for 5 min. Slides were dried on a slide warmer at 37ºC for 10 min, fixed with absolute methanol, and stained with Diff-Quik stain (Dage Diagnostics, Aguada, Puerto Rico).

2.1.5 Single Cell Gel Electrophoresis (SCGE) The procedure for the SCGE assay followed that reported by Tice et al. [10] with minor modifications. V79 cell culture and sample treatment for SCGE assay was the same as that for micronucleus assay. After challenge, cells were rinsed with phosphate-buffed saline (PBS, Gibco) and harvested by trypsinization. The cell suspensions were centrifuged at 600 x g at 4ºC for 10 min and the cell pellets were resuspended in 0.5 ml of complete medium (approximately 1.5 - 2 x 106/ml). Tubes containing the resuspended cells were kept on ice for making sandwich gel slides.

Electrophoresis was carried out at 300mA and approximately 25 volts (0.7 v cm^{-1}) for 30 min at room temperature. Slides were transferred to Coplin Jars, soaked in Tris buffer for 5 min three times to neutralize the gel. The slide was then stained with 50 µl of 2 µg/ml ethidium bromide (Sigma-Aldrich) and covered with a cover slip for 5 min, followed by rinsing in distilled water. The finished slides were covered with a cover slip and stored in a humidified airtight container at 4ºC for analysis. Image analysis was performed at 200x magnification using a fluorescence microscope (Zeiss, 7082 Oberkochen, Germany) equipped with a 100 W Hg vapor lamp, a 515 – 560 nm excitation filter and a 590 nm emission filter. The tail length of DNA migration was determined with an eyepiece micrometer by measuring the distance between the edge of the nucleus to the end of the tail. For each treatment, 25 cells per slide were analyzed for 4 replicate slides.

3. Results

3.1. Mutagenicity

Results of the salmonella gene mutation test are shown in Table 1, and data for the assay using the YG1024 tester strain without S9 activation are graphed in Figure 1, showing response as revertants per plate, while concentration values in the Table and Figure are in terms of the mass of SRM 2975 DPM dispersed for the DPM-DPPC dispersed samples and in terms of mass of SRM 2975 DPM subjected to extraction for the DPM-Solvent Extract samples. Regression equations and corresponding p-values are given in Table 2 for linear fits to the concentration-response data. Both DPM acetone solvent extract and DPM dispersion in DPPC showed statistically significant positive mutagenic activity to both YG1024 and YG1029 strains both with and without S9 activation; and YG1024 gave a stronger mutagenic response than YG1029 to both DPM solvent extract and DPM dispersion in DPPC samples The mutagenic activity in general, was stronger without S9 metabolic activation. Overall, activity for DPM dispersion in DPPC was stronger than that of DPM solvent extract samples.

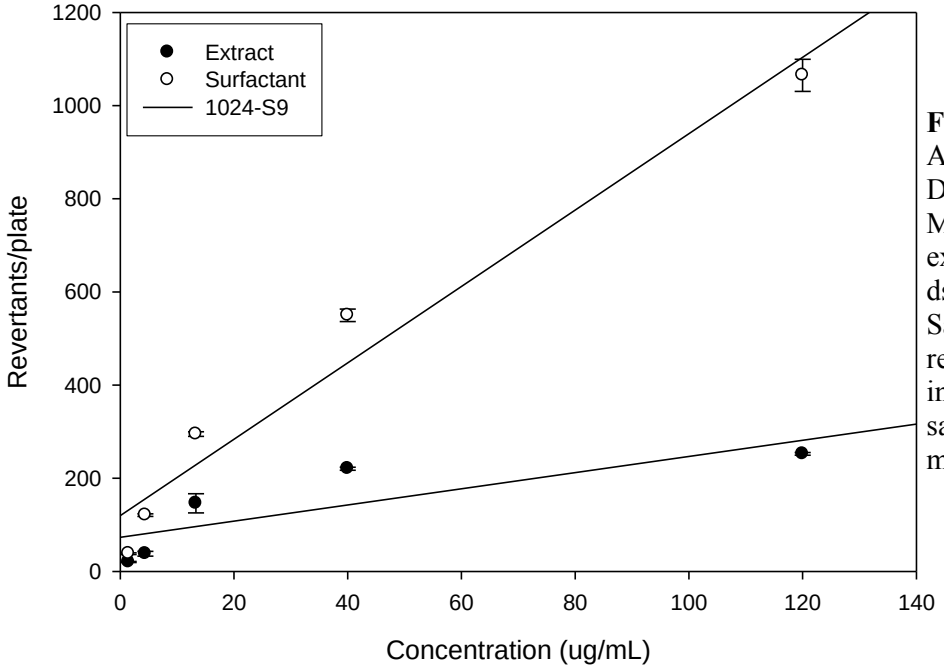

Figure 1: Mutagenic Activity of SRM 2975 Diesel Particulate Material as solvent extract or as surfactant dspersion using Salmonella YG1024 reversion to histidine independence, without sample activation by S9 microsomal enzyme.

4

Table 1: Revertant Colonies Induced by Diesel Particle Material (DPM) SRM 2975 in Salmonella Typhimurium YG1024 and YG1029

Test Sample	Concentration[a] (µg/plate)	Average number of revertant colonies/plate[b] YG1024		YG1029	
		w/out S9	with S9	w/out S9	with S9
DMSO	10[c]	18 ±2	29±5	27± 4	26±6
DPM Solvent Extract	13.3	146 ±51	24±3	63± 1	51±5
DPM Solvent Extract	40	221± 8	77±4	152± 23	79±7
DPM Solvent Extract	120	252± 7	435±32	351± 11	192±2
DPPC	10[c]	23 ±1	41±5	62± 3	79±2
DPM-DPPC	13.3	295 ±13	167±3	173± 3	99±13
DPM-DPPC	40	550± 33	456±65	357± 25	188±16
DPM-DPPC	120	1065± 84	1007±81	750± 60	560±9
2AA[d]	0.05				794±16
1-NP[e]	0.05	403±10		428±2	

[a] Concentration based on original DPM subjected to extraction or dispersion.

[b] Average of 6 counts. [c] µl/plate. [d] 2-aminoanthracene. [e] 1-nitropyrine.

Table 2: Regression Equations for Salmonella mutagenic activity in Revertants/plate versus sample concentration in terms of original SRM2975 extracted or dispersed

	Extract	Surfactant	p-value
1024+S9	y = -11.84 + 3.57x	y = 64.14 + 8.06x	< 0.0001
1024-S9	y = 76.37 + 1.70x	y = 119.86 + 8.20x	< 0.0001
1029+S9	y = 34.81 + 1.29x	y = 53.94 + 4.13x	< 0.0001
1029-S9	y = 27.07 + 2.74x	y = 89.91 + 5.62x	< 0.0001

3.2. Micronucleus Induction

Results of the comparative study of micronucleus induction in V79 cells, based on equal concentration of SRM 2975 DPM subjected to acetone extraction or DPPC dispersion, are given in Table 3 and plotted in Figure 2. DPM solvent extract induced significant micronucleus formation at concentrations of 180µg/ml ($P<0.05$) and 240 µg/ml ($P<0.01$). DPM dispersion in DPPC induced significant micronucleus formation with positive concentration-response behavior for all of the concentrations tested. Micronucleus formation activity in V79 cells was stronger for surfactant-dispersed whole soot particles than for the organics extracted from an equal mass of soot. Tables 3, 4 and 5 and Figure 2 also show the results of micronucleus assay of DPPC-dispersion of the solid residue of acetone solvent-extracted SRM 2975 DPM, and of DPPC-dispersed ultrafine carbon black. The residue of acetone-extracted SRM 2975 was inactive for micronucleus induction when assayed as a dispersion in DPPC. Carbon black, assayed as an ultrafine particulate material bearing no extractable genotoxicants, also was inactive for micronucleus induction as a dispersion in DPPC.

Table 3: Comparative Studies of Micronucleus Induction by Diesel Particle Material (DPM) SRM 2975 in V79 Cells

Test sample	Concentration[a] (µg/ml)	MNC/1000 cells[b] (Mean±SD)
DMSO	5[c]	5.6± 2.3
DPM Solvent Extract	40	6.3± 1.6
DPM Solvent Extract	180	7.6± 2.3*
DPM Solvent Extract	240	9.9± 2.2**
DPPC		5.8 ±1.9
DPM-DPPC	40	7.2± 2.3*
DPM-DPPC	180	11.1 ±2.8**
DPM-DPPC	240	16.1 ±2.2**
DPM Solvent Residue-DPPC	40	6.5± 1.7
DPM Solvent Residue-DPPC	180	6± 3
DPM Solvent Residue-DPPC	240	5.2± 2
Carbon Black-DPPC	40	5.6± 2.6
Carbon Black-DPPC	180	6.6± 3.3
Carbon Black-DPPC	240	5.6± 1.8
MNNG[d]	0.25	23.4± 2.8**

[a] Based on original DPM extracted or dispersed. [b] Micronucleated cell counts were based on 12000 cells scored. [c] µl/ml. [d] N-methy-N-nitro-nitrisoquanine. * $P \leq 0.05$ compared with negative control. ** $P \leq 0.01$

Additional micronucleus induction assays were performed comparing the activity of a mass of SRM 2975 DPM dispersed in DPPC with an equal mass of extract obtained by acetone extraction of a (larger) mass of SRM 2975 DPM. Shown in Table 4 and Figure 3, both preparations caused significant micronucleated cells at all such concentrations ranging from 13.3 to 180 μg/ml, and both exhibited a concentration-dependent increase in activity.

Table 4: Regression Equations for number of micronucleated cells versus concentration in terms of original SRM2075 DPM extracted or dispersed.

Extract:	y = 5.44 + 0.016x
Surfactant:	y = 5.25 + 0.041x
Carbon Black:	y = 5.72 + 0.001x
Residue:	y = 6.83 – 0.006x

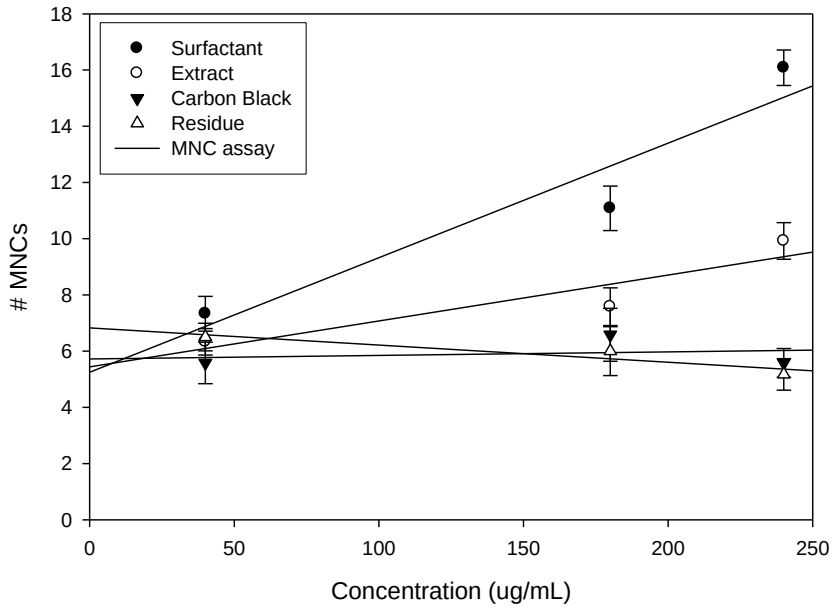

Figure 2. Micronuclei Induced by SRM2979 DPM dispersed in DPPC surfactant or extracted by acetone; by carbon black dispersed in DPPC; and by DPPC dispersion of the residue of acetone extraction of DPM.

Table 5: Induction of Micronucleus by Diesel Particulate Material SRM 2975 in V79 Cells

Test sample	Concentration[a] (μ g/ml)	MNC/1000 cells[b] (Mean±SD)	Multiple Nucleus cell/1000 cells (Mean±SD)
DMSO	5[c]	5.5 ±2.3	11.8± 3.4
DPM Solvent Extract	13.3	15.7± 2.2**	22.8± 3.1**
DPM Solvent Extract	40	17.5 ±2.1**	26.7 ±3.8**
DPM Solvent Extract	120	21.7± 6.3**	25.0 ±7.1**
DPM Solvent Extract	180	32.5 ±2.8**	39.8 ±5.7**
DPPC		7.8± 2.5	18.6± 3.0
DPM-DPPC	13.3	9.8 ±3.5	26.2± 8.2*
DPM-DPPC	40	12.2 ±1.6**	39±.3 4.6**
DPM-DPPC	120	15.3± 4.1**	34.2± 12.6**
DPM-DPPC	180	19.3 ±3.6**	57.0± 13.7**
NC		7.5± 2.6	15.5± 1.9
MNNG[d]	0.25	29.7± 4.2**	11.5 ±2.5

[a] For solvent extraction the concentration was based on extracted material, for DPPC dispersion the concentration was based on original DPM dispersed. [b] Micronucleated cell count (MNC) was based on 1000 cells. A total of 6000 cells were scored. [c] μl/ml. [d] N-methy-N-nitro-nitrisoquanine * $P< 0.05$ compared with negative control. ** $P < 0.01$ compared with negative control.

Table 6: DNA damage Induced by Diesel Particle Material (DPM) SRM in V79 Cells with SCGE

Test Sample	Concentration[a] (μg/ml)	Damaged Cells/ 100 Cells	Tail Length (μ m)[b] (Mean±SE)
DMSO	5[c]	14	1.1± 0.28
DPM Solvent Extract	13.3	20	1.1± 0.25
DPM Solvent Extract	40	22	1.5 ±0.29
DPM Solvent Extract	120	33	2.4± 0.37**
DPM Solvent Extract	180	54	2.6± 0.27**
DPPC		13	1.2 ±0.32
DPM-DPPC	13.3	10	0.8± 0.25
DPM-DPPC	40	29	2.1 ±0.34*
DPM-DPPC	120	38	2.6 ±0.36**
DPM-DPPC	180	41	2.9± 0.37**
Complete Medium	0	9	0.7± 0.21
MNNG[d]	0.25	89	8.1± 0.35**

[a] For solvent extraction the concentration was based on mass of extracted material, for DPPC dispersion the concentration was based on mass of original DPM dispersed. [d] N-methy-N-nitro-nitrisoquanine. [b] Based on 100 cells. [c] μl/ml * $P< 0.05$ ** $p< 0.01$ vs. control.

3.3. DNA damage

Results from the SCGE assay of DNA damage in V79 cells were scored as tail length (TL) in μm. Assays were performed comparing the activity of a mass of SRM 2975 DPM dispersed in DPPC with an equal mass of extract obtained by acetone extraction of a (larger) mass of SRM 2975 DPM. Any cell with a nonzero tail was classified as damaged cell. Both exposure of V79 cells to DPM solvent extract and to DPM dispersion in DPPC caused significant DNA migration at concentrations ranging from 120 to 180 and 40 to 180 μg/ml, respectively and displayed a concentration-dependent increase in tail length in Table 6 and Figure 2. The percentage of damaged cells increased with concentration-response manner in both DPM solvent and dispersion in DPPC.

Table 7: Logistic Regression Equations for number of DNA-damaged cells /100cells versus concentration in terms of original SRM2075 DPM extracted or dispersed.

Extract:	$y = 1.6248 – 0.0092x$
Surfactant:	$y = 1.6217 – 0.0079x$

4. Discussion

Numerous in vitro genotoxicity studies have been performed upon diesel exhaust particulate materials (DPM) collected by filtration and assayed as organic solvent extracts. In many but not all cases such extracts have been found to express positive genotoxic activities. However, the physiologically anomalous use of organic solvent extracts of DPM questions the biological availability of DPM-borne genotoxicants under conditions of deposition in the lung. And such conventional in vitro genotoxicity assays applied to DPM organic solvent extracts cannot identify the possible effects of exhaust particulate size or structure on genotoxic activity.

We have reported that some DPM can express in vitro genotoxic activities as mixtures into aqueous dispersion of some principal phospholipid pulmonary surfactants, e.g., dipalmitoyl phosphatidyl choline, DPPC. Extraction of DPM by DPPC does not result in significant genotoxic activity by the extract; however, the non-dissolved particulate phase of direct mixture of DPM into DPPC dispersion can express in vitro genotoxic activities. Results suggest that particles adsorb surfactant onto their surfaces from aqueous dispersion, making the so-conditioned particle surfaces hydrophilic and permitting their dispersion in aqueous media. This retains particle structure and its effects on the expression of toxicities, partially modeling particle surface conditioning upon inhalation and deposition in the deep lung. This permits the application of in vitro bioassays in a manner which avoids anomalous test preparation destruction of particle size and structure, factors which may be important in the expression of toxicities by ultrafine or nano-particles. Further, it partially models the effects on DPM toxicity of the conditioning of respirable particle surfaces which occurs promptly upon respirable particle deposition in the deep lung onto the surfactant-rich aqueous hypophase coating of the respiratory bronchioles and alveoli. Surfactant dispersion concentration requirements for this have been determined in terms of particulate surface area [11]

The current study objective was to compare some in vitro genotoxic activities between solvent-extraction and surfactant- dispersion preparations for a generally available diesel exhaust reference material, U.S. National Institute for Standards and Technology DPM standard reference material SRM 2975. Assays were chosen to represent bacterial mutagenicity and mammalian cell chromosomal or DNA damage.

In Salmonella mutagenicity assays using either of two tester strains, both solvent extract and surfactant dispersions of the DPM were active, expressing for at least one sample concentration more than twice the solvent or surfactant control activity, and demonstrating a positive concentration – mutagenic activity response. Comparing mutagenic activities versus concentrations measured on the basis of mass of original DPM dispersed into DPPC or mass of original DPM subjected to solvent

extraction, it was found that the surfactant dispersion preparation was significantly more active for a given concentration than the solvent extract.

Those, similarly, were the results for micronucleus induction in mammalian-derived V79 cells: both surfactant dispersion and solvent extract preparations of SRM 2975 DPM were active, with surfactant dispersion being significantly more active than the solvent extract for concentration based upon mass of DPM extracted or dispersed. Comparison also was made using the micronucleus assay to identify a possible contribution to the activity from the presence of non-dissolved particles per se in the surfactant dispersion preparations. This was approached by assaying a carbon black particulate material in surfactant dispersion, and by assaying in surfactant dispersion the non-dissolved particulate residue surviving organic solvent extraction of SRM2975 DPM. Surfactant dispersions of both types of insoluble particles carrying no extractable genotoxicants were inactive. Micronucleus induction also was compared between the two preparations also on the basis of mass of original SRM 2975 DPM dispersed in surfactant versus equal mass of solvent extracted DPM material (from a larger amount of original SRM 2975 DPM). In that comparison, a mass of extracted material is significantly more active than an equal mass of dispersed DPM. It is noted that a given mass of extract requires the extraction of a much larger mass of DPM for this SRM2975 DPM with solvent extractables of about 10% the mass of the DPM extracted.

DNA damage was compared between the two preparations only on the basis of concentrations of surfactant dispersed SRM 2975 DPM versus equal concentrations of material extracted from a (larger) amount of SRM 2975 DPM. In this SCGE assay under this manner of concentration comparisons, there was no significant difference in activity between the two preparations. Again, with the low solvent extractable fraction of this SRM 2975 DPM, this suggests surfactant dispersion of this DPM is at least as active and reasonably more active than the solvent extract of an equal mass of original SRM 2975 DPM.

The implication of these results is that for this SRM 2975 DPM, a given mass of extractable DPM-associated genotoxic materials express greater in vitro genotoxic activities if they are associated with surfactant-dispersed particles than is expressed by the genotoxicants when extracted from the particles. Lack of in vitro genotoxic activity of surfactant-dispersed carbon black and of surfactant-dispersed SRM2975 which had been stripped of genotoxic compounds by acetone extraction, suggest the greater activities seen for this DPM in surfactant dispersion may be a synergistic effect of particle-genotoxicant association rather than an additive effect of activity of the carried genotoxicants plus activity of carrier genotoxicant-barren particles.

That not all DPM solvent extracts express genotoxic activity; and that diesel engine design and operational factors affect the amount of emissions and specific genotoxic activities of DPM solvent extracts, has suggested that in vitro assays of DPM might be a source of convenient first-tier guidance for diesel design or exhaust control technology development with the objective of minimizing hazardous diesel exhaust emissions. The results of in vitro assays of surfactant-dispersed DPM suggest that such efforts can be extended to include a more physiologically-relevant preparation of DPM for in vitro testing which is not blind to considerations of the size and structure of DPM and its conditioning upon deposition in the lung. Our current research includes extension of the findings of in vitro activities of surfactant-dispersed DPM to the concept of collection of exhaust DPM directly into surfactant dispersion for bioassay.

References

[1] International Agency for Research on Cancer, 1989. Diesel and gasoline engine exhausts and some nitroarenes. Vol. 46. Monographs on the Evaluation of Carcinogenic Risks to Humans. World Health Organization, IARC, Lyon, France

[2] US Environmental Protection Agency, 2002. Health Assessment Document for Diesel Engine Exhaust. USEPA EPA/600/8-90/057F. 01 May 2002. U.S. EPA, Office of Research and

Development, National Center for Environmental Assessment, Washington, DC.

[3] McMillian MH., Cui M, Gautam M, Keane M, Ong T, Wallace W, Robey E [2002]. Mutagenic Potential of Particulate Matter from Diesel Engine Operation on Fischer-Tropsch Fuel as a Function of Engine Operating Conditions and Particle Size. Soc.Auto.Engineers Technical Paper 2002-01-1699, pp. 1-18.

[4] Wallace WE, Keane MJ, Hill CA, Xu J, Ong TM [1987]. "Mutagenicity of Diesel Exhaust Particles and Oil Shale Particles Dispersed In Lecithin Surfactant". Journal of Toxicology and Environmental Health, 21 163-171.

[5] Wallace W, Keane M, Murray M, Chisholm W, Maynard A, Ong T . "Phospholipid Lung Surfactant and Nano-Particle Surface Toxicity: Lessons from Diesel Soots and Silicate Dusts. Journal of Nanoparticle Research 9(1): 23-38.

[6] King, LC., Kohan MJ, Austin AC, Claxton LD, Hunsingh JL [1981]. Evaluation of the release of mutagens from diesel particles in the presence of physiological fluids. Environ. Mutag. 3:109-129.

[7] Ames, B., McCann J, Yamasaki E [1975]. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res. 31: 347-364.

[8] Countryman PI and Heddle JA [1976]. The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. Mutat Res. 41(2-3):321-32.

[9] Roberts CJ, Morgan GR, Holt PD [1986]. A critical comparison of the micronucleus yield from high and low LET irradiation of plateau-phase cell populations. Mutat Res. 160(3):237- 242.

[10] Tice RR, Strauss GSH,Peters WP [1992]. High-dose combination alkylating agents with autologous bone-marrow support in patients with breast cancer: preliminary assessment of DNA damage in individual peripheral blood lymphocytes using the single cell gel electrophoresis assay. Mutat. Res. 271:101-113.

[11] Wallace WE, Keane MJ, Gautam M, Shi X-C, Murray D, Ong T "Dispersion of Nanoparticles in Pulmonary Surfactants for In Vitro Toxicity Studies: Lessons from Ultrafine Diesel Exhaust Particles and Fine Mineral Dusts". In; Nanotoxicology: Characterization, Dosing, and Health Effects on Target Organs, Edited by N. Monteiro-Riviere & L. Tran; Informa Healthcare USA, Inc., New York. ISBN-10: 1-4200-4514-8. Chapter 11; pp. 153-171.