DIESEL EXHAUST DISPERSION

IN A PHOSPHOLIPID LUNG SURFACTANT

FOR RETENTION OF NANO-PARTICULATE STRUCTURE

IN SHORT-TERM BIOASSAYS

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Notes

This is a review of our findings that diesel exhaust particulate matter (DPM) can express genotoxic activities in bacterial or mammalian cells when the DPM is dispersed into a major component, phospholipid,of the surfactant that lines the surface of the deep lung airways and air sacs.

Some results are presented from the published literature of National Institute for Occupational Safety and Health (NIOSH) research; and some current research is discussed.

The method and findings suggest a way to perform short-term *in vitro* or instillation *in vivo* toxicity tests which model a potentially critical step in respirable particle exposure: the adsorption of lung surfactant onto particle surfaces and the subsequent effects on biological availability of particle-borne toxicants under conditions of deposition in the lung.

Diesel Exhaust Exposures and Genotoxicity

Probable or likely human carcinogenic risk

(International Institute for Research on Cancer-IARC,

US Environmental Protection Agency - EPA, NIOSH)

NIOSH-National Cancer Institute study underway:

Mortality Study of Lung Cancer and Diesel Exhaust Among Non-Metal Miners

Tumorigenic mechanisms ?

- Genetic
- Epigenetic
- Nano-particulate (size < 0.1 micrometers) effects

Organic Solvent Extracts of Some DPM are Genotoxic

Some DPM contain ultrafine particle-bound organic compounds which, separately, are genotoxic *in vitro* or tumorigenic *in vivo*

Organic solvent extracts
of some DPM →

- bacterial cell mutagenicity
- mammalian cell DNA damage
- mammalian cell chromosomal damage

Lung Surfactant Extracts of DPM are Not Genotoxic

Lung surfactant (e.g., phospholipid) extracts of some DPM

→ little or no *in vitro* genotoxicity

Are DPM-borne organic genotoxicants biologically available for activity in the lung?

Notes

Although DPM sometimes contain compounds that are separately genotoxic or tumorigenic in some animal model tests, there is a question of the “biological availability” of particle–bound genotoxicants to express activity.

That is, while some of the compounds are mutagenic when tested after extraction from DPM “soot” particles by organic solvent, e.g., acetone or dichloromethane, are those compounds able to express genotoxic activity under conditions of deposition in the deep lung respiratory bronchioles or pulmonary alveoli?

At first the answer would seem to be “no”; several studies have found that lavaged lung surfactant or phospholipid models of lung surfactant do not extract (dissolve and so release) materials with genotoxic activity from filter-collected DPM.

Finding:
Some DPM are Genotoxic in Lung-lining Surfactants

Some whole diesel exhaust particulate material

when dispersed in

(not extracted by)

some surfactants that coat the deep lung

induce *in vitro* genetic damage:

- Mutation in bacterial cells
- DNA damage in mammalian cells
- Chromosomal damage in mammalian cells

Notes

However, NIOSH research has found that *in vitro* genotoxic activities are expressed by some DPM, not when the DPM is formally extracted by lung surfactant, but instead when DPM is mixed whole into surfactant;

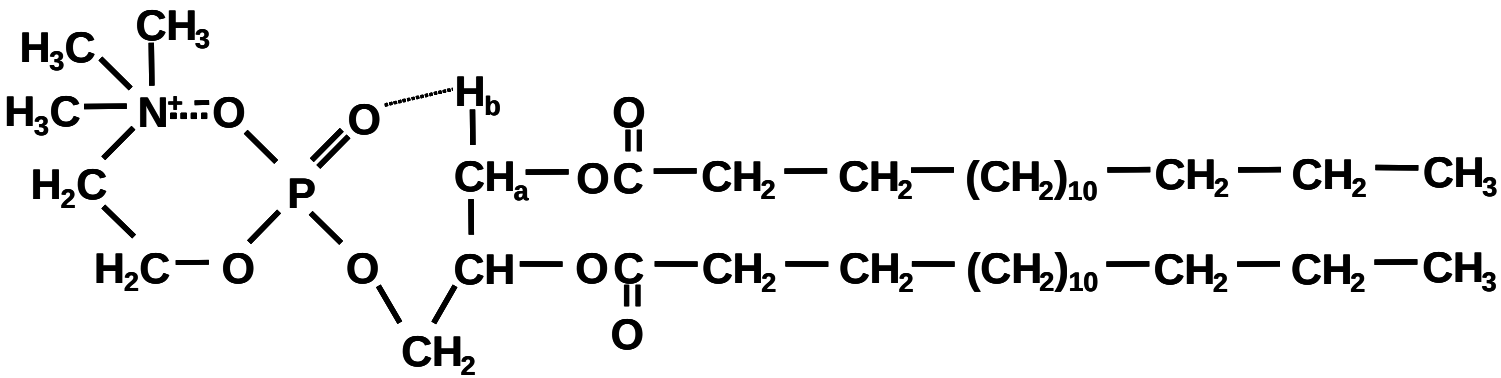
that is, when DPM is dispersed in surfactant as fine or ultrafine particleswith an adsorbed surfactant coating on the particles.

This, to first order, models the prompt initial conditioning

of fine or ultrafine particles depositing on the surface of the deep lung. Such “physiological” conditioning permits particle-borne materials to express genotoxic activities in bacterial cells and mammalian lung-derived cells.

DPPC Surfactant Structure:

Palmitate residues associate with DPM hydrocarbon;
zwitterionic phosphatidyl choline head orients outward
→ adsorbed molecular conformation provides a
"wettable" surface on a surfactant-coated DPM particle



Notes

Surfactants are synthesized and secreted onto the wet surface of the deep lung by alveolar type II cells. Phospholipids are a major component of lung surfactant.

By themselves they can reproduce physiologically-important surface-tension properties of the pulmonary alveolar hypophase surface.

We principally use dipalmitoyl phosphatidyl choline (DPPC) dispersed into physiological salt-concentration sterile saline (PSS) to model lung surfactant.

The long chain lipophilic/hydrophobic tails of the DPPC molecule associate with organic particle surface, while the electrically-charged trimethyl ammonium and phosphate groups on the head of the molecule are hydrophilic, and orient outward to face the surrounding aqueous medium.

Notes

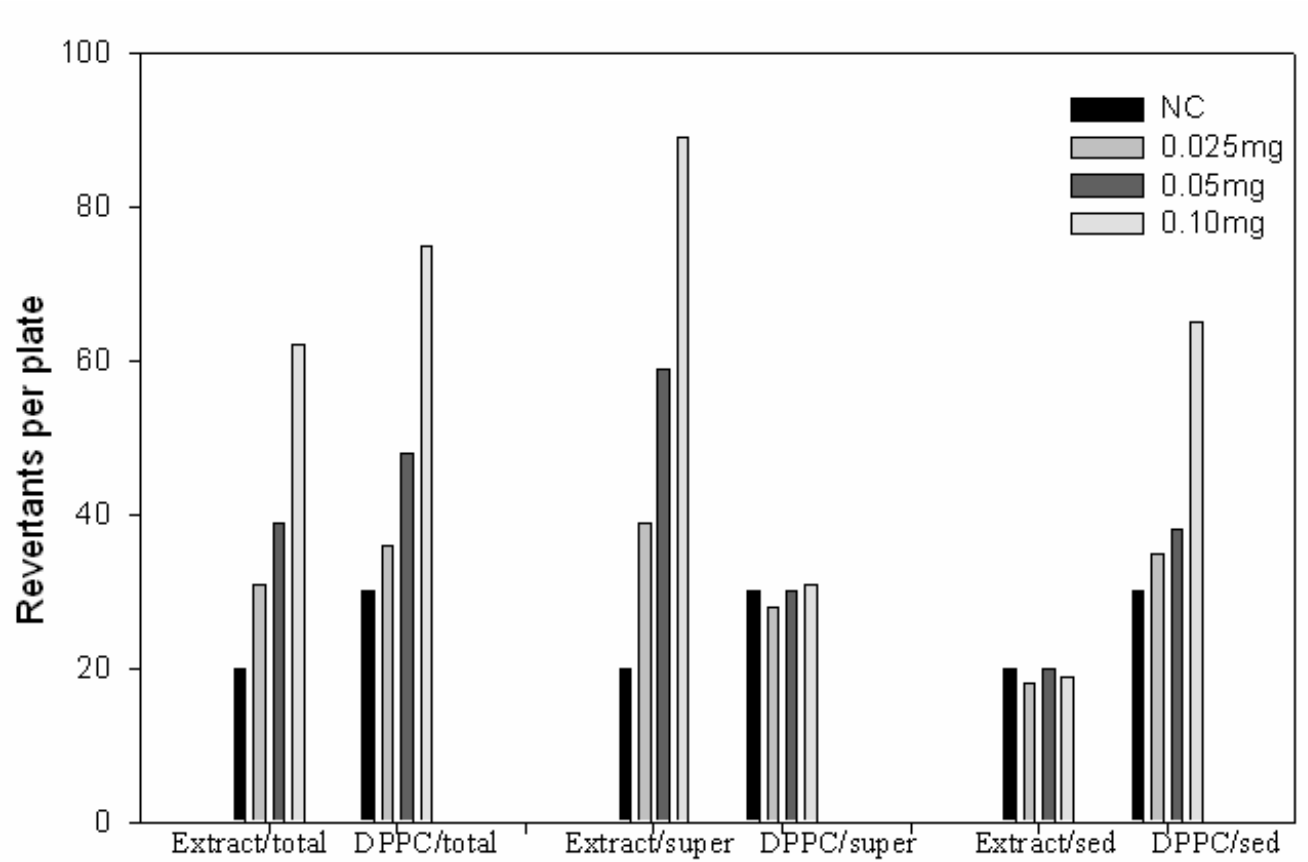
Thus, the molecule orients itself such that the long fatty acid residue chain tails adsorb to the particle surface and the hydrophilic head groups are oriented to be in contact with the surrounding water molecules.

A simplified picture is a DPM particle as a tar “pin-cushion” covered by DPPC soap molecules with their tails stuck as the shaft of pins to the pin-cushion and their heads outward, providing a hydrophilic outer coating, in-turn permitting the structure to act as a water-wet but non-dissolved fine- or nano-particle which disperses in water.

These small “solubilized” DPM particles in surfactant dispersion can express genotoxic activities.

Mutagenic Activity (TA98 Salmonella Bacterial Cell) Versus DPM Concentration:

Solvent extract or DPPC surfactant dispersion: total preparation, filtered supernatant, sediment



Notes

Salmonella mutagenicity (revertants/plate) versus total or fractionated preparations of DPM as organic solvent extract or as surfactant dispersion:

Test Preparation

Cells: *Salmonella typhimurium* TA98 without S9 microsomal activation.

Sample: Filter-collected DPM supplied by Lovelace-ITRI: 1980 GM 5.7 liter V8 engine run on the Federal Test Procedure Urban Driving Cycle.

Surfactant: DPPC ultrasonically dispersed in PSS; (2.5 mg DPPC/ 1 ml PSS).

Solvent preparations of DPM: Dissolved DPM in dichloromethane (DCM), evaporative exchange into dimethylsulfoxide (DMSO (1 mg DPPC/ 1 ml DMSO).

Surfactant preparations of DPM: Mixed DPM into surfactant/PSS dispersion (1 mg DPM / 2.5 mg DPPC / 1 ml PSS).

Test materials

(a) Total preparations;

(b) supernatant from centrifugation and filtration of total preparations (dissolved/extracted material);

(c) sediment from centrifugation of total preparations (non-dissolved particulate phase material).

Test protocol

90 min pre-incubation of 0.025, 0.05, 0.1 mg DPM in 0.1 ml solvent or PSS plus 0.5 ml PSS plus 0.1 ml TA98 @ 1-2 $x10^8$ cells/ml;

then mixed with 2 ml top agar / 0.05 mM biotin + histidine; grew cells 48 h;

count colonies.

Notes

Results/Interpretation

Total dispersion: solvent and surfactant total preparations are comparably mutagenic.

Supernatant fraction (extracted material):
Solvent preparation supernatant was positive;
Surfactant preparation supernatant was negative,
i.e., no active mutagenic material extracted from DPM by surfactant.

Sediment fraction (non-dissolved particulate material):
Solvent preparation was negative, i.e., the carbonaceous residue of solvent extracted DPM was not mutagenic;
Surfactant preparation was positive, i.e., the particulate matter which is not dissolved by surfactant is nevertheless, positive for mutagenic activity as a particulate dispersion in surfactant.

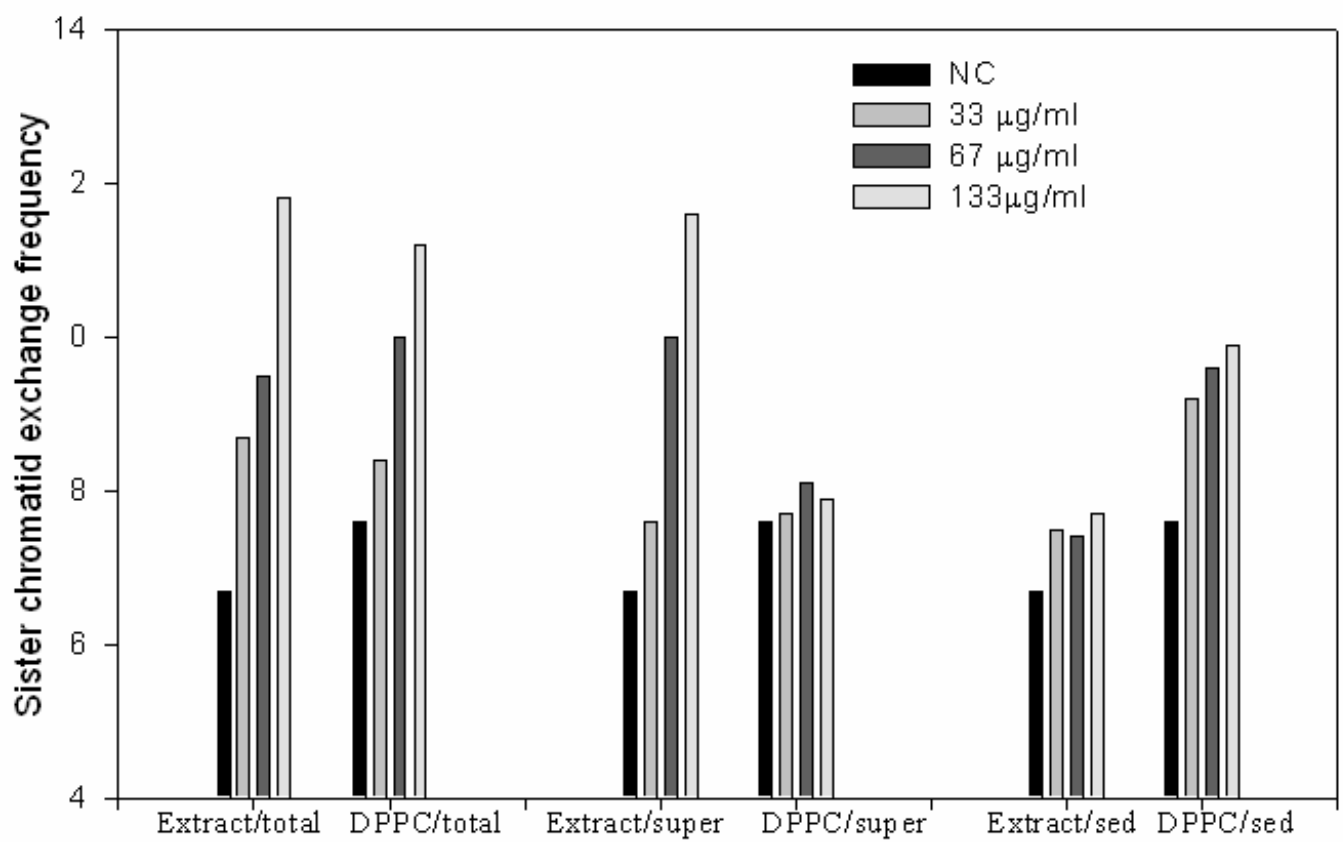
Comments

Other tests of the surfactant preparation supernatant fraction using only centrifugation without subsequent filtration resulted in some activity in the surfactant preparation supernatant. This suggests an ultrafine (nano-) particulate fraction of DPM dispersed in surfactant that was mutagenic.

Mutation Research 260: 233-238, 1991.

Sister Chromatid Exchange (V79 Mammalian Cell) :

Number/cell versus DPM concentration; solvent extract or DPPC surfactant dispersion; total preparation, filtered supernatant, sediment



Notes

Sister chromatid exchange frequency per cell in V79 mammalian cell-line versus DPM concentration for total or fractionated preparations of DPM as solvent extract or surfactant dispersion.

Test preparation

Cells: Chinese hamster pulmonary fibroblast-derived cell line V79.
Sample: DPM was the same as that used for the bacterial mutation test.
Surfactant: 2.5 mg DPPC per ml PSS.
Solvent preparation: Extract from DCM was exchanged into DMSO (10 mg DPM/1 ml DMSO).
Surfactant preparation = 10 mg DPM / 2.5 mg DPPC / 1 ml PSS.

Test materials

(a) Total sample;
(b) centrifugation/filtration supernatant;
(c) centrifugation residue.

Notes

Test protocol

2 -3 X 10^6 cells in a flask containing 15 ml medium were challenged with 33, 67, or 113 micrograms DPM/ml medium for 5 h; added BrdU; incubated 36 h; fluorescence stained; read 25 cells as M2 chromosome spreads.

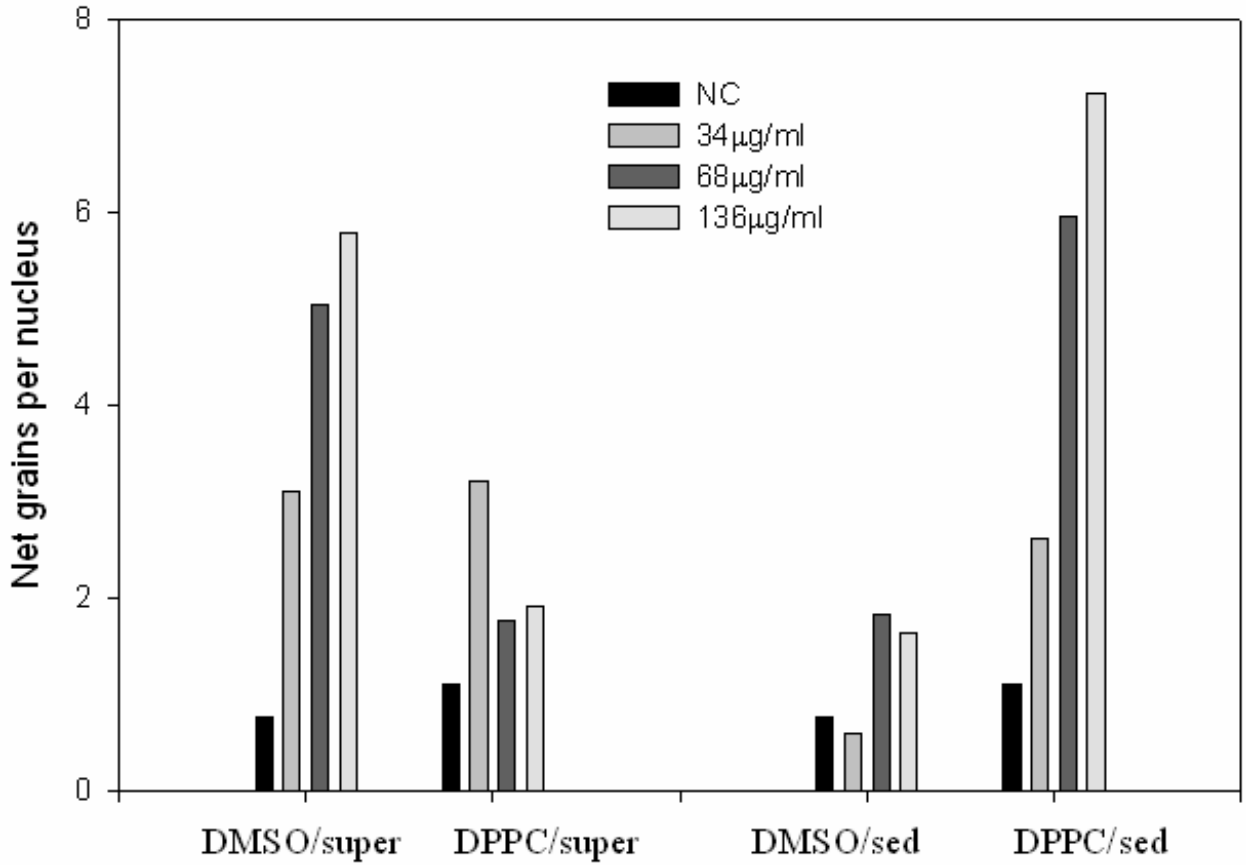
Results/Interpretation

Clastogenic activity expressed by DPM organic solvent-dissolved material and by DPM surfactant-dispersed particulate material.

Mutation Research 260: 233-238, 1991.

Unscheduled DNA Synthesis (V79 Mammalian Cell) :

Net grains/nucleus versus DPM concentration; solvent extract or DPPC surfactant dispersion; total preparation, filtered supernatant, sediment



Notes

Unscheduled DNA synthesis in V79 mammalian cells: ^{3}H-thymidine autoradiography net nuclear grains (NNG) versus DPM concentration, for DPM solvent extract or surfactant dispersion particulate material.

Test preparation

Cells: V79.

Sample: DPM was the same as that used for the bacterial mutation test.

Solvent preparations of DPM: DCM extract of DPM was dissolved in DMSO.

Surfactant: 10 mg DPPC per ml PSS.

Surfactant DPM dispersion: 10 mg DPM / 10 mg DPPC / 1 ml PSS.

Test materials

(a) Total sample
(b) centrifugation/filtration supernatant;
(c) centrifugation residue.

Notes

Test protocol

Approximately 1X10^6 cells were treated with ^{3}H-thymidine and 34, 68, 0r 136 micrograms DPM/ml medium for 16 h.
After hypotonic treatment, slides were prepared and autoradiography was performed.
An average NNG equal to or greater than 5 was considered too be a positive response.

Results/Interpretation

Mean NNG were significant for the two higher concentrations for total solvent or surfactant preparations, for solvent extract, and for surfactant-dispersed particulate phase material.

Annals of Occupational Medicine 38(1):345-349, 1994.

Chromosomal Aberrations (V79 Mammalian Cell) :

Percent aberrant cells versus DPM concentration; DPPC surfactant total dispersion

Treatment	Concentration µg/ml	Aberrant Cells (%)	Total Aberrations [a] With Gaps	Total Aberrations [a] Without Gaps
DPPC	0.1	10	10	3
D7 in DPPC	25	27[b]	28[b]	14[b]
D7 in DPPC	50	35[cb]	38[b]	18[b]
D7 in DPPC	100	33[b]	43[b]	19[b]
D7 in DPPC	150	43[b]	52[b]	27[b]
MNNG[c]	1	62[b]	95[b]	67[b]

[a] 100 metaphases scored
[b] $p<0.01$ by $\chi 2$ test
[c] N-methyl-N-nitro-N-nitrosoguanidine

Notes

Chromosomal Aberrations in V79 cells: percent aberrant cells versus DPM concentration in surfactant dispersion:

Test preparation

Cells: V79 cells.
Sample: DPM sample was the same as that used for the bacterial mutation tests.
Surfactant: 10 mg DPPC per ml PSS.
Surfactant dispersion preparation of DPM: 10 mg DPM / 10 mg DPPC / 1 ml PSS.

Test protocol

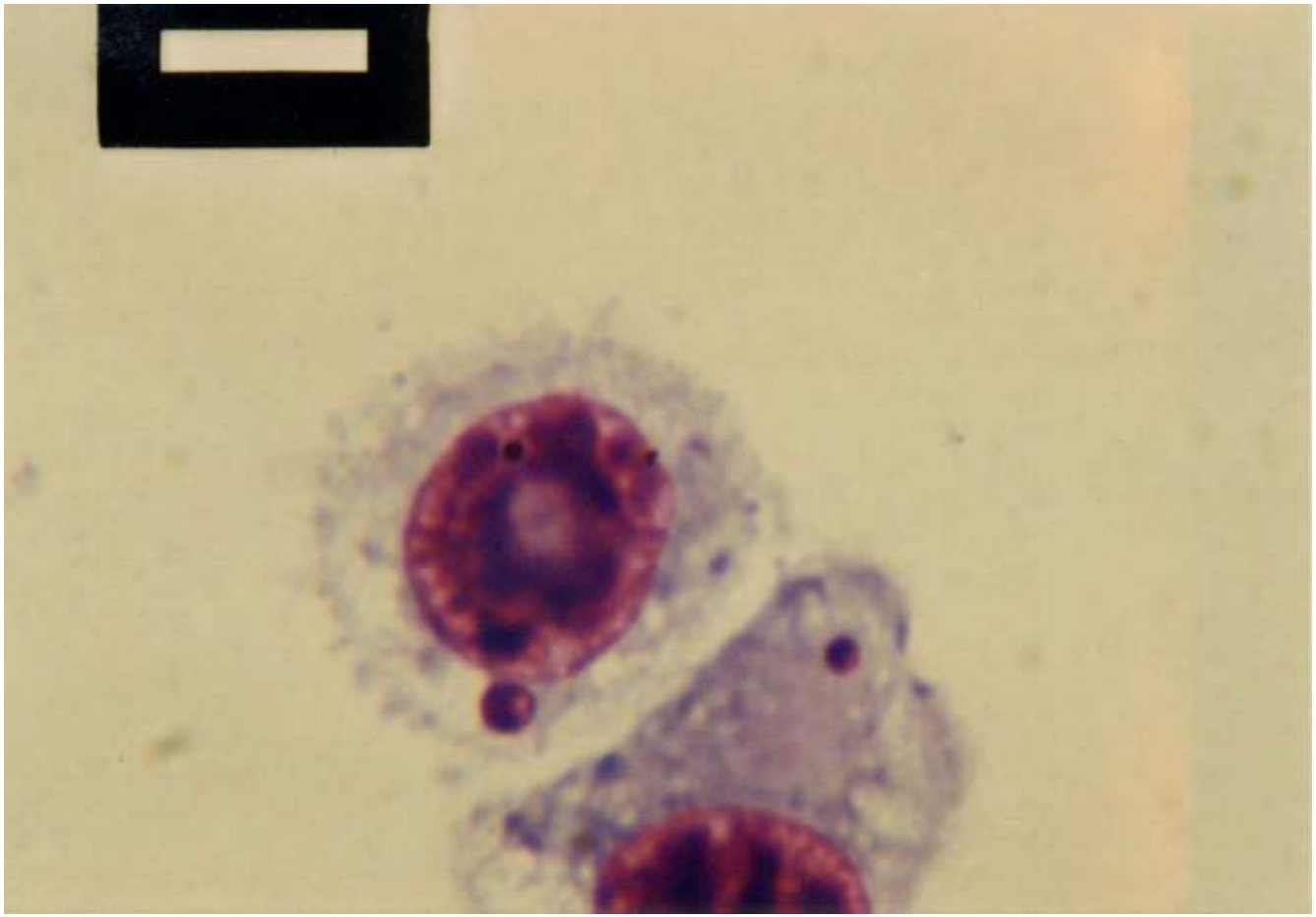
2X106 cells in 5 ml medium were treated with 125, 250, 500, or 750 microgram of DPPC-dispersed DPM for 48 h. Cells were reseeded 10^6 cells for 22 h; colcemid was added and cells were stained. 100 cells in M2 metaphase each containing 19-23 chromosomes were analyzed. Chromosomal aberrations scored included gaps, breaks, minutes, terminal deletions, accentric rings, dicentric chromosomes, interchanges, aneuploidy, polyploidy.

Results/Interpretation

surfactant-dispersed DPM induced chromosomal aberrations.

Journal of Toxicology & Environmental Health 68: 431-444, 2005.

Micronuclei in V79 cells



Micronucleus Induction in V79 or CHO Mammalian Cells

frequency per 500 cells versus DPM concentration; solvent or surfactant filtered supernatant or sediment

Treatment	Concentration(ug/ml)	Micronucleus frequency (mean +/- SD)	
		V79	CHO
DMSO	5	18.3 +/- 2.1	9.3 +/- 0.8
Supernatant	34	25.2 +/- 2.6	18.7 +/- 3.4
	68	29.3 +/- 5.6	17.2 +/- 4.6
	136	37.3 +/- 4.8	23.8 +/- 3.3
Sediment	34	23.5 +/- 5.1	6.0 +/- 0.9
	68	23.2 +/- 5.4	10.0 +/- 3.6
	136	21.5 +/- 6.2	8.0 +/- 2.2
DPPC	5	21.5 +/- 5.0	7.2 +/- 1.7
Supernatant	34	21.2 +/- 5.2	11.5 +/- 5.1
	68	20.4 +/- 11.1	10.0 +/- 2.7
	136	22.0 +/- 5.6	9.8 +/- 3.0
Sediment	34	23.8 +/- 3.1	18.5 +/- 4.3
	68	32.7 +/- 3.4	19.0 +/- 3.7
	136	23.2 +/- 8.8	20.5 +/- 2.3
MNNG	1	81.7 +/- 11.4	214 +/- 29.7

Notes

Micronucleus induction in V79 or CHO cells versus DPM concentration for DPM solvent extract or surfactant dispersion supernatant and particulate phase materials:

Test preparations

Cells: Chinese hamster pulmonary fibroblast-derived (V79) cells and ovary-derived (CHO) cells.
Sample: DPM was the same as that used for the bacterial mutation tests.
Surfactant: 10 mg DPPC per ml PSS.
Solvent preparation: Extract from DCM was exchanged into DMSO (10 mg DPM/1ml DMSO).
Surfactant preparation: 10 mg DPM/ 2.5 mg DPPC/1 ml PSS.

Test materials

(a) Centrifugation supernatant
(b) centrifugation residue (sediment).

Notes

Test protocol

Approximately 2X 10^6 cells were treated with 170, 340, 780 microgram of DPM in 5 ml medium for 24 h. Cells were further incubated for 24 h without DPM. Slides were prepared and cells were stained. Scored 3000 cells for micronucleus from each treatment.

Results/Interpretation

The solvent supernatant (extract) of total sample after centrifugation and filtration, and the surfactant sediment (particulate material) from total sample centrifugation were active for micronucleus induction in CHO cells: the solvent extract was active in V79 cells, but the surfactant sediment was only marginally active in V79 cells.

Comment:
current testing of a National Institute of Standards and Technology (NIST) standard diesel particulate material indicates positive micronucleus activity in V79 cells.

Mutation Research 279: 55-60, 1992.

Single Cell Gel Electrophoresis (SCGE) of Damaged Cellular DNA ("Comet" Assay)

Notes

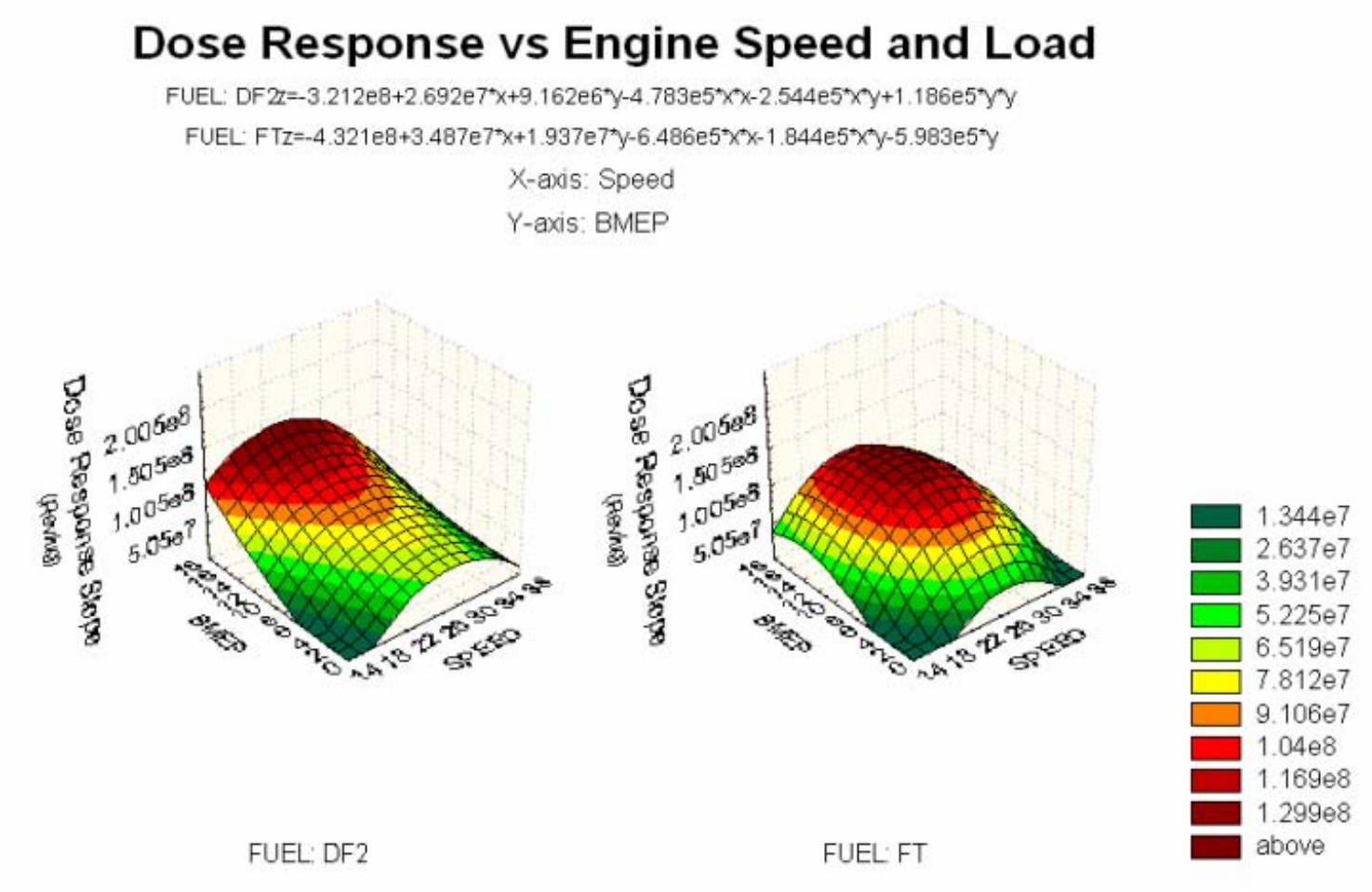
Research is underway testing DNA damage induction in V79 cells as measured by single cell gel-electrophoresis tailing of damaged DNA from individual cells (the “comet” assay).
Total and sediment/supernatant fractions of solvent or surfactant dispersions of a National Institute for Standards and Technology (NIST) standard diesel exhaust particulate sample are being assayed.

DPM Extract Genotoxic Activity is a Function of Engine/Fuel/Operation

Mutagenic activity
of diesel particulate solvent extract is
a function of engine operating conditions,
e.g., rpm, loading, injection timing

Bacterial Mutagenicity of DPM Solvent Extract Versus Diesel Engine Speed and Load

McMillian MH, et al., Society of Automotive Engineers Technical Paper 2002-01-1699, pp. 1-18.



Notes

From:
“Mutagenic Potential of Particulate Matter from Diesel Engine Operation on Fischer-Tropsch Fuel as a Function of Engine Operating Conditions and Particle Size”. McMillian MH, Cui M, Gautam M, Keane M, Ong T, Wallace W, and Robey E. In: Society of Automotive Engineers Technical Paper 2002-01-1699, pp. 1-18. (2002).

DPM chemical and physical properties are well-known to be functions of design and operational parameters: engine design, mode of operation, state of tuning, fuel, lubrication oil, …
These factors also determine the potential genotoxicant activity of DEP.

Here the filter-collected DPM from a single-cylinder stationary diesel engine, operated at a set of steady-state speed and load points, in tests at the Dept. of Energy – National Energy Technology Laboratory, result in differing bacterial mutagenic activities (computed from salmonella mutagenicity testing of filter-collected DPM organic solvent extract using two strains of *Salmonella typhimurium* with and without S9 microsomal activation, in assays performed at NIOSH).

Notes

Comment:

Similar maps of genotoxic activities versus engine/control system design and operational parameters can be constructed for surfactant-collected or dispersed DPM.

In vitro bacterial or mammalian cell genetic toxicology end-points or in vivo instillation animal model studies can be designed to use DPM directly collected into pulmonary surfactants, or to use filter-collected DPM which is then dispersed into pulmonary surfactants.
This addresses concerns for the physical state of DPM nano-particulate material and the biological availability of DPM particle-associated geno-toxicants, as they would be manifest upon inhalation exposure in an occupational/environmental setting.

With the rapid evolution of diesel emission control technologies for improved emissions control, this information might inform engineering initiatives and assist the selection of representative DPM from prototype engine/control systems for longer-term animal model inhalation testing.

Other Observations:

- Tests with particulate residue of organic solvent extracted DPM = no activity → particles without genotoxicants were inactive
- Tests with supernatant of centrifuged vs filtered (<0.2 micrometer) DPM dispersion in surfactant → significant activity associated with ultrafine particles
- Tests of DPM dispersion in other phospholipid surfactants (dipalmitoyl phosphatidyl ethanolamine -DPPE, dipalmitoyl phosphatidic acid - DPPA) → comparable bacterial mutagenic activity to same DPM dispersed DPPC
- Mammalian cell 6-thioguanine-resistant gene mutation not induced by solvent- or surfactant-preparation of DPM.
- Surfactant adsorption suppresses some particulate cytotoxic activities (non-genetic toxicity) → possibly an aid to the expression of genotoxic activity

Conclusions / Discussion:

- Genotoxic activity is expressed *in vitro* by some DPM dispersed in a primary lung surfactant.
- This suggests a mechanism providing for biological-availability of some DPM geno-toxicants under conditions modeling early deposition in the lung,
- and provides a method for assaying *in vitro* genotoxic activity in a manner modeling initial physiological conditioning and biological-availability of particles depositing in the deep lung.
- Geno-toxicant content and *in vitro* genotoxic activity of solvent extract of DPM is a function of engine system / fuel / operation.
- New engine system/operation parameters controlling DPM *in vitro* genotoxic activities similarly can be determined for surfactant-collected or dispersed DPM to better model nano-particle structural effects and toxicant biological availability in the lung.

Elements of a Strategy

→ DPM collection/ physiological short-term bio-assay as a dispersion in lung surfactant

→ (using a standardized set of assays, e.g., *Salmonella* reversion for mutagenicity; mammalian cell micronucleus induction for chromosomal damage; mammalian cell SCGE for DNA damage)

→→ Identify critical chemical/physical properties of DPM affecting biological activities

→→→ Identify engine/ exhaust control system factors determining DPM critical properties/toxicities

→→→→ inform engineering development;

→→→→ inform selections for longer-term animal model testing

References: *In Vitro* Genotoxicity of DPM Dispersed in Phospholipid Surfactants

"Mutagenicity of Diesel Exhaust Particles and Oil Shale Particles Dispersed In Lecithin Surfactant".

Wallace WE, Keane MJ, Hill CA, Xu J, Ong TM, in Journal of Toxicology and Environmental Health, 21 163-171 (1987).

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