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MUTAGENICITY OF DIESEL EXHAUST PARTICLES AND OIL SHALE PARTICLES DISPERSED IN LECITHIN SURFACTANT

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Diesel exhaust particulate material from exhaust pipe scrapings of two trucks, diluted automobile diesel exhaust particulate material collected on filters, and two oil shale ores were prepared for the Ames mutagenicity assay by dichloromethane (DCM) extraction, by dispersion into 0.85% saline, or by dispersion into dipalmitoyl lecithin (DPL) emulsion in saline. Salmonella typhimurium *TA98 was used to detect frameshift mutagens in the samples. Samples of diesel soot gave positive mutagenic responses with both DCM extraction and DPL dispersion, with the DPL dispersion giving higher results in some cases. The results suggest that possible mutagens associated with inhaled particles may be dispersed or solubilized into the phospholipid component of pulmonary surfactant and become active in such a phase.*

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INTRODUCTION

The Ames *Salmonella*/microsome assay (Ames et al., 1975) has been shown to be a useful test for detecting genotoxic properties of various agents. Hundreds of specific chemicals have been screened (McCann et al., 1975), and the test has been applied for many types of environmental samples. Airborne mutagenic agents, for example, have been found in urban air (Chrisp and Fisher, 1980; Hughes et al., 1980; Rosenkranz et al., 1978), in diesel engine exhausts (Huisingh et al., 1978), and in the byproducts of coal combustion processes (Chrisp et al., 1978).

Samples of airborne particles are typically collected on membrane filters, and undergo a rigorous extraction procedure with organic solvents and/or ultrasonic agitation (Clark and Hobbs, 1980). Often extracts are transferred to another solvent that is compatible with the assay system. These extraction procedures, while efficient from an analytical perspective, are not physiologically plausible. The questions that present themselves are whether a demonstrated mutagen can be taken up in vivo and whether the process is quantitatively or qualitatively different from the above laboratory procedure (Wallace et al., 1984).

When a particle of respirable size is inhaled to the alveolar region of the lung, its first physiological contact is with pulmonary surfactant that lines the inner surfaces of the alveoli. This surfactant is an aqueous fluid containing proteins, phospholipids, and other components (R. J. King, 1982). The principal phospholipid constituent is L-alpha-phosphatidylcholine, beta,gamma-dipalmitoyl, or dipalmitoyl lecithin (DPL) (L. C. King et al., 1981).

The objectives of this study were to determine whether mutagenic compounds can be transferred to a fluid representative of the phospholipid component of pulmonary surfactant and whether the results of mutagenicity testing following such preparation are comparable to the results of preparatory procedures using organic solvents. These objectives were intended to address the question of in vivo biologic availability of mutagenic hydrophobic respirable particles deposited in the acinar region of the lung and to test a preparatory procedure for mutagenicity assays of respirable particles that takes account of that question (Wallace et al., 1986).

The fluid used was dipalmitoyl lecithin emulsified in physiological saline. Several substances not expected to exhibit mutagenic activity were also assayed to screen for possible false positive results.

MATERIALS AND METHODS

Diesel soot (D1) was obtained by scraping the inside of the exhaust pipe of a large diesel truck at the West Virginia University Medical

Center. The soot was collected immediately after the engine was stopped. Magnetic particles were removed with a magnet. Diesel soot (D2) was provided by Dr. Trent Lewis of the National Institute for Occupational Safety and Health (NIOSH) Division of Biomedical and Behavioral Science. The soot was generated by a stationary diesel engine, and was over 1 yr old at the time used. The particulate matter was scraped from the inside of filter bags. Dusts D1 and D2 were stored in sterile jars at −10°C. Diesel soot (D3) was provided courtesy of Dr. Roger McClellan of the Lovelace Inhalation Toxicology Research Institute (ITRI). Exhaust particulate material was collected by high-volume sampling on filters in a dilution tunnel diluting the exhaust of a General Motors 5.7-l engine operated on a Federal Test Procedure Cycle, and the filter was stored at −80°C. Paraho oil shale (OS1) and Scottish oil shale (OS2) were provided by Dr. James Jackson of Los Alamos National Laboratory (LANL). The ores were ground to fine powders with mortars and pestles, and stored in sterile vials at −10°C.

Dipalmitoyl Lecithin Emulsion, 10 mg/ml

For this preparation, 100 mg L-alpha-lecithin, beta,gamma dipalmitoyl (DPL) (Calbiochem, La Jolla, Calif.) was weighed into a sterile 50-ml centrifuge tube, 10 ml of 0.85% physiological sterile saline (PSS) was added, and the liquid was sonicated 10 min at 40 W (Heat Systems-Ultrasonics, Plainview, N.Y.). The probe was wiped with 95% ethanol and was covered with Parafilm during the sonication.

Dichloromethane Extraction

For this, 2.5 mg of each particulate material was suspended in 2.5 ml of dichloromethane (DCM) (Mallinckrodt, St. Louis, Mo.) in sterile 15-ml glass centrifuge tubes and sonicated in a water bath (Bransonic, Shelton, Conn.) for 30 min at room temperature. Tubes were placed in a dry bath at 45°C, 2.7 ml dimethyl sulfoxide (DMSO) (Baker, Philipsburg, N.J.) was added, and the DCM was evaporated under nitrogen, until 2.5 ml of liquid remained.

DPL Dispersion

For this, 2.5 mg of each particulate material was weighed into a sterile glass centrifuge tube, 2.5 ml of 10 mg DPL/ml saline was added, and the tube was placed in a water bath for 60 min at 37°C. PSS dispersion was similar to DPL dispersion, except PSS was used.

Samples extracted with DCM were diluted 1:2 and 1:4 with DMSO; samples dispersed with DPL or PSS were diluted likewise with PSS. Particulate matter was not removed in any of the assays except the experiments involving sedimentation, as described below. Negative controls were DMSO, PSS, and DPL. Positive controls were technical-grade trinitrofluorenone (TNF) (Aldrich, Milwaukee, Wis.), 1 mg/ml, for experi-

ments without S9, and 2-aminoanthracene (2AA) (Aldrich, Milwaukee, Wis.), 2.5 mg/ml, for the experiment with S9.

For experiments that involved separation of dispersed or extracted diesel soot into a liquid phase and a sedimented phase, samples were centrifuged 10 min at 2000 × g after the extraction or dispersion procedure. The supernatant was removed and diluted as above, and the sediment resuspended in either DMSO or PSS, vortexed, and diluted as above. For experiments that involved filtration of the supernatant from centrifugation of dispersed or extracted diesel soot, samples were first dispersed or extracted and then centrifuged as above. The supernatant was then filtered through an 0.2-μm syringe filter (Millipore, Bedford, Mass.). The filtrate was then diluted as above for assay.

The Ames *Salmonella*/microsome assay was used for mutagenesis testing, and the S9 mixture was prepared as described by Ames et al. (1975). The preincubation test was used as the assay system. In brief, 0.1 ml of each sample, 0.5 ml PSS or S9 mixture, and 0.1 ml of TA98 culture was added to a 16 × 100-mm plastic culture tube, and all sample tubes were rotated in a 37°C incubator for 90 min. Samples were then mixed with 2 ml top agar supplemented with biotin (0.05 m*M*) and histidine (0.05 m*M*), and mixtures were poured into Vogel–Bonner minimal media plates and incubated 48 h at 37°C. Revertant colonies were counted with an electronic counter (Artek, Farmingdale, N.Y.); plates containing visible diesel particles were hand counted to assure that only *Salmonella* colonies were counted. Since diesel soots D1 and D2 had been shown by previous work in this laboratory to be mutagenic to TA98 without S9, this tester strain was used exclusively, and S9 activation was used in only one experiment.

RESULTS

Figure 1 shows the results for D1 for each of three preparation media: dichloromethane, physiological saline, and dipalmitoyl lecithin emulsified in saline. Mutagenic response increases with dose in all cases. The responses of DPL-dispersed samples and the DCM-extracted samples both substantially exceed the response of the saline-dispersed samples. The results from duplicated experiments show that the response in revertants per plate is generally higher for DPL-dispersed than for DCM extracted samples. An additional experiment was done to see the effect, if any, of S9 microsomal fraction on the samples prepared by the three methods. The S9 fraction supressed the mutagenic response in all the samples, especially those dispersed in DPL. Potential reasons for this have not yet been investigated. The S9 fraction also elevated the controls. This is not unexpected, noting that some histidine may be present in the liver homogenate.

As shown in Fig. 2, diesel soots D2 and D3 both met the criterion for mutagenicity (response producing an increase equal to two or more

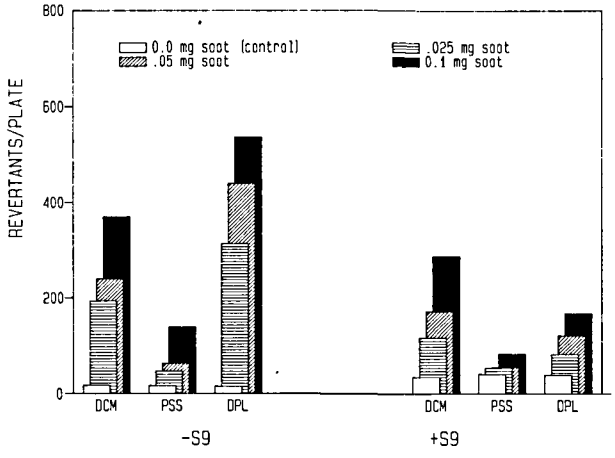

FIGURE 1. Mutagenic response versus extraction or dispersion medium for diesel soot D1 with and without S9 microsomal activation.

times the control for at least one concentration tested, and a positive dose related response at three concentrations) (Ames et al., 1975). The responses from OS1 and OS2 were similar for all three preparation media; OS1 minimally meets the criterion for mutagenicity with DCM extraction, but otherwise the responses for all preparation media were very weak. The mutagenic responses of both positive and negative controls were typical for the TA98 tester strain in all experiments.

To examine whether DPL was acting as an extracting agent or as a

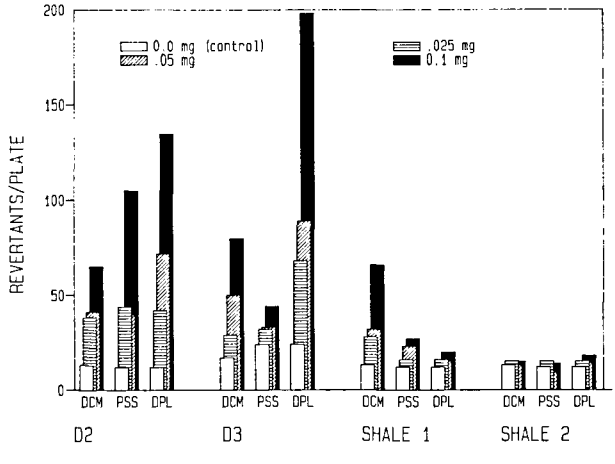

FIGURE 2. Mutagenic response versus extraction medium for diesel soot D2 and D3, and oil shales OS1 and OS2.

solubilizing or dispersing agent in this system, samples were centrifuged 10 min at 2000 × g after the DCM extraction or DPL dispersion procedures. The supernatants were transferred to vials, and the sediments were resuspended in DMSO or saline, respectively, for the DCM-extracted or DPL-dispersed samples. Figure 3 shows that virtually all of the mutagenic activity for the DCM-extracted samples is in the supernatant, but the greater activity lies with the sediment phase in the DPL-prepared samples. To investigate whether the mutagenic activity in the supernatant was due to dissolved or dispersed soot fractions, a set of samples were subjected to this same procedure of DCM extraction or saline or DPL–saline dispersion and subsequent centrifugation. The supernatant fraction from the centrifugal separation was filtered through a 0.2-μm filter. The filtrates were then assayed. Figure 4 shows that this filtration removed all mutagenic activity from the DPL–saline-prepared sample supernatants.

An experiment was performed to determine the effect of varying DPL emulsion concentration (the concentration of dipalmitoyl lecithin emulsified in saline) on the mutagenic response of diesel soot D1 dispersed in the emulsion. Figure 5 shows that the response increases with increasing DPL concentration up to 7 mg DPL/ml saline, where the effect levels off.

Another experiment showed that mutagenic response is essentially constant for unseparated sample D1 incubated for times of 1, 2, 4, and 8 h in dispersion in 10 mg DPL/ml saline at 37°C; the dispersion times do not include the 90 min associated with the preincubation procedure.

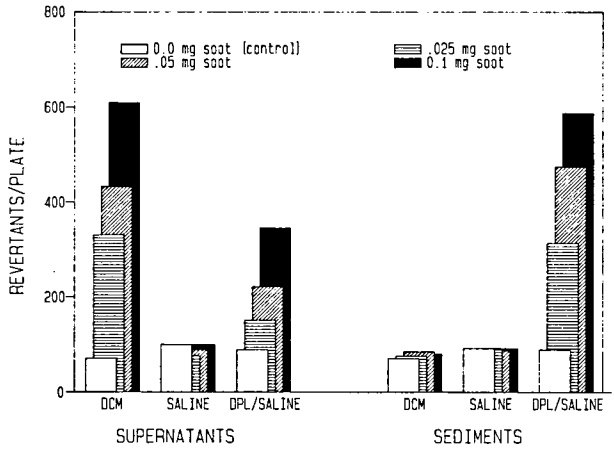

FIGURE 3. Mutagenic response for supernatant and 2000 × g sediment phases of diesel soot D1 versus extraction or dispersion medium.

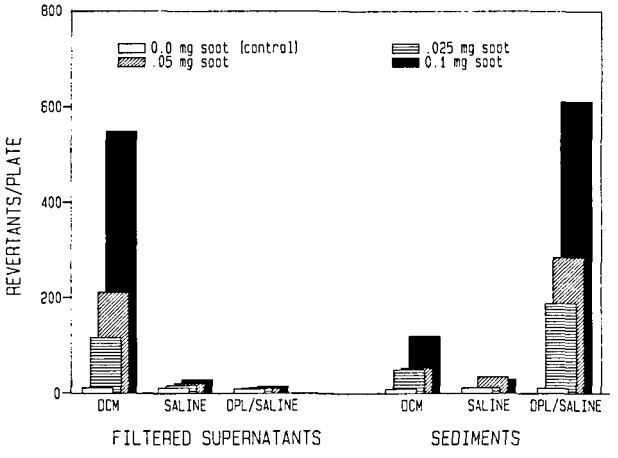

FIGURE 4. Mutagenic response for filtered supernatant and 2000 × g sediment phases of diesel soot D1 versus extraction or dispersion medium.

DISCUSSION

From this study it seems clear that mutagenic agents from some diesel soots can express mutagenic activity when dispersed in phospholipid emulsion that resembles a major component of pulmonary surfactant. It is especially surprising that the response in many cases is greater than that using extraction with organic solvents. This seemingly is contradictory to the findings of L. C. King et al. (1981) and Brooks et al. (1981), who found, respectively, mild response with lung lavage

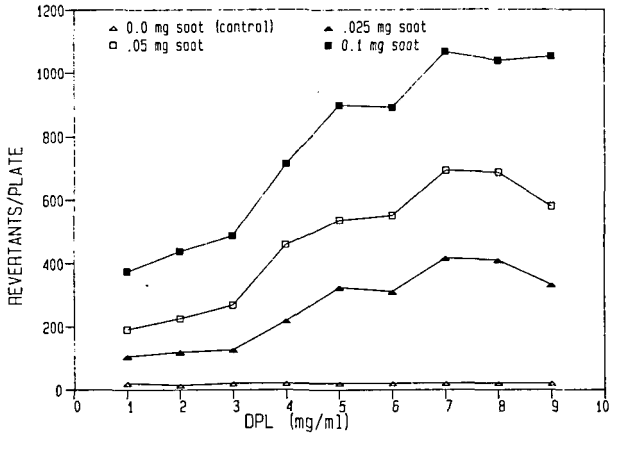

FIGURE 5. Mutagenic response verus lecithin concentration in emulsion for diesel soot D1, without S9 activation.

fluid extracts and no response with both lavage fluid and DPL extracts of diesel particulate material. However, one crucial difference between this work and those studies is that in this work particulate materials were not removed by filtration or sedimentation before the assay. Their studies used several hours to several days of incubation with extractants, but this would not appear to be the basis for the differences in results (L. C. King et al., 1981; Brooks et al., 1981).

In the experiment where samples were separated into a supernatant and a sedimented phase, both fractions were assayed. After 2000 × g centrifugation, most of the mutagenic activity was found in the sediment for DPL-dispersed samples, in contrast to DCM-extracted samples. Filtration of the supernatants removed the remaining mutagenic activity from the DPL-dispersed sample supernatant. It therefore appears that the supernatant-fraction mutagenicity of the DPL-dispersed samples is due to very fine dispersed particles, which might be removed by the separation procedures used in other studies (L. C. King et al., 1981; Brooks et al., 1981). DPL may be solubilizing the diesel soot, rather than extracting a soluble fraction.

Since the majority of mutagenic response with DPL-dispersed samples is with the sedimented material, this is evidence that the DPL may facilitate interaction of diesel soot particles with the *Salmonella* auxotrophs. Microscopic examination of D1 revealed most particles were less than 1 μm in diameter, consistent with published data on other diesel exhaust particulate material (Cheng et al., 1984). This is very fine but still sizeable relative to bacteria (less than 2 μm in length). Since the TA98 auxotrophs have cell-wall defects (Ames et al., 1973), these defects may allow contact of the plasma membrane with particles or agents adsorbed on particles.

These results suggest a research premise that components of pulmonary surfactant provide a solubilization mechanism for hydrophobic or sparingly water-soluble respirable particulate material that deposits in the acinar regions of the lung. Such solubilization might affect one or more steps in the chain of exposure from contact to uptake to metabolism and interaction with genetic material within the cell.

In any case, this sample shows that surrogate physiological fluid is capable of efficient delivery of mutagenic agents from particles to tester cells. Additional research is needed to elucidate the mechanisms involved.

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