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Assay of Mutation Induced in Human Lymphoblastoid Cells by Combustion-Generated Soot Particles

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A human lymphoblastoid cell line has been used to test for mutations caused by combustion-generated soot particles and their constituent components, which are substrate carbon-black and adsorbed condensate, principally in the form of polycyclic aromatic hydrocarbons (PAH). It was found that the mutagenicity of the PAH fraction is higher when it is contacted with cells as a liquid extract than when it is supplied as a coating on soot particles. The substrate particles were found to be nonmutagenic. The rate of transfer of mutagens from the surface of particles, combined with the retention time of respirable aerosol particles, are deemed to define their mutagenic potential.

Introduction

A number of polycyclic aromatic hydrocarbons (PAH) formed during fuel combustion processes, such as benzo[*a*]pyrene and cyclopenta[*c,d*]pyrene, have been identified as potent mutagens in tests with human lymphoblastoid cells (*1*). Such compounds are formed from smaller molecules that are produced during the incomplete combustion of hydrocarbon fuels. Upon cooling of the combustion gases, PAH condense onto soot (carbon black) particles that are also generated during incomplete combustion, as has been described by many investigators [e.g., Homann and Wagner (*2*)]. Consequently, soot has been identified as a carrier of carcinogens and thus as a potential health hazard.

It is less clear, however, to what extent the particle–adsorbate complex is more or less mutagenic than the constituent elements and how the size, morphology, and other physical characteristics of soot particles affect the release (bioavailability) of mutagens in the organism. In other words, the nature of the mass transport processes involved in transferring potentially mutagenic materials from particle surfaces to the inside of cells where mutations may be induced are not well known. Crespi et al. (*3*), using a line of metabolically competent human lymphoblastoid cells found that the efficiency of bioextraction of mutagens adsorbed onto soot particles was lower (~13%) than that of the same materials dissolved in the culture medium in the form of an extract. On the other hand, inhalation studies conducted by Bond and co-workers on animals with PAH adsorbed on particles have shown that this association of the mutagens with solid particles increases the long-term pulmonary retention of PAH compounds and their metabolites and augments their carcinogenicity in the lung (*4*). Similar results have been reported by Wolff et al. based on inhalation studies with aerosols of benzo[*a*]pyrene (BaP) and the same substance adsorbed on carbon black particles (*5*).

Lakowicz, Bevan, and Riemer (*6,7*) investigated the effect of adsorption onto particle matter of several PAH on the rate of their uptake in model membranes formed by lipid bilayers. Their results show increased rates of membrane uptake of PAH adsorbed on particles of several inorganic materials when compared to aqueous suspensions of microcrystals of the same substances. However, in one test conducted with carbon black (soot) particles, it was observed that no detectable amount of adsorbed benzo[*a*]pyrene was released over comparable time periods. These results show the critical effect of particle surface properties on the rate of release and, hence, the toxicity and mutagenicity of the adsorbed materials.

Risby and co-workers (*8–10*) modeled and quantitatively measured thermodynamic and kinetic parameters governing the transfer (adsorption and desorption) of aromatic and heterocyclic compounds to and from carbon black particles in contact with a liquid phase consisting of a liposomal solution that simulates alveolar surfactant. Their results show that the desorption of such molecules, and hence their bioavailability, is controlled by the presence of active sites on the surface of carbon black particles that selectively absorb and retain adsorbate molecules. This selectivity is in turn influenced by the characteristics of the solvent. Release is also shown to depend on the degree of surface

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coverage of the particles. Wallace and co-workers (*11,12*) showed a critical role for alveolar surfactants in enhancing the mutagenic activity of diesel exhaust particles of respirable size in bacterial mutation assays. They suggest that the pulmonary surfactant coating assists the transport of respired insoluble particles and thus enhances genotoxic activity in the acinar regions of the lung.

The mechanism generally proposed for the introduction of toxic materials from the atmosphere to the organism is by alveolar deposition of solid particles of "respirable" size (approximately 0.1–5.0 μm), which are either cytotoxic in their own right (e.g., quartz, asbestos) or are carriers of adsorbed toxic or mutagenic materials (e.g., combustion aerosols). In this latter case a further transfer of material from the particle surface to the cell must occur before the mutagenic activity of the substance in question can be expressed. This transfer may occur by one or more mechanisms: direct contact between particles and cells and transfer across cell membranes, engulfing of particles by cellular processes such as phagocytosis, or dispersion of the toxic or mutagenic substance by dissolution and transport by diffusion. The net rate of release of mutagens adsorbed onto inhaled particle surfaces is a likely critical parameter affecting their potency.

The present paper reports initial results obtained on the mutagenic effect of soot particles generated in a well-characterized and controlled combustion process and using a sensitive line of lymphoblast human cells for exposure. The experiment concerns the measurement of the relative bioavailability of PAH adsorbed onto soot particles.

Materials and Methods

Cells

The MCL-3 derivative of AHH-1 TK+/− cells was used in this study. This cell line is a derivative of the MCL-1 cell line described by Davies et al. (*13*) and expresses human cytochrome P-4502A3, P-4501A2, and microsomal epoxide hydrolase cDNAs. Cells were cultured in RPMI medium 1640 supplemented with 9% v/v of horse serum. Hygromycin B (100–200 μg/mL) was added to maintain selection for the cDNA-containing vector.

Soot Particles and PAH Adsorbate

Soot particles were generated in a premixed (ethylene/oxygen/nitrogen) laminar flame burner with a carbon-to-oxygen mole ratio of 0.83 (fuel equivalence ratio of 2.49). Combustion products, including soot particles, were withdrawn isokinetically using a water-cooled stainless-steel probe with a characteristic quench time of about 12 msec located 2.0 cm above the burner. Under the conditions of the experiment, this corresponds to an in-flame residence time of approximately 2 msec. The soot particles were collected on a 0.1-μm pore membrane filter (Durapore; polyvinylidene dichloride). The soot particle surface area and diameter were determined from *in-situ* light scattering and absorption measurements. The soot particle surface area so measured was found to be 55 m^2/g. This value for the specific surface area was subsequently confirmed by BET measurements on collected soot particles. The geometric mean diameter of primary particles was calculated to be approximately 50 nm. The equipment and methodology for generating, collecting, and characterizing the soot samples have been described in detail by Feitelberg (*14*).

To determine the amount of extractable condensate on the soot particles, the particles were extracted by sonication in methylene dichloride. The sonication was done for 20 min in a Bronson-450 sonifier set at 40 watts and a 100% duty cycle. The resulting extract was separated from the stripped particles with an 0.1-μm Durapore membrane placed in a syringe filter. The amount of stripped particles was determined by differential weighing of the membrane filter, and the extract was weighed by evaporating the methylene dichloride from three separate aliquots of the extract placed in aluminum pans weighed before and after the process, as has been described by Lafleur et al. (*15*). The relative amounts of substrate and extracted adsorbate for soot obtained from three different burner runs under nominally identical conditions are shown in Table 1. The extract was analyzed by gas chromatography with a flame ionization detector to identify its principal known constituents. The compounds so identified and their relative amounts are listed in Table 2.

Mutation experiments were conducted with soot particles, stripped paticles, and extract from soot particles. The mutation experiments with soot particles were conducted with and without the addition of controlled amounts of dimethyl sulfoxide (DMSO), which is used to solubilize the PAH in the aqueous medium of cell cultures. The extract samples were obtained by sonicating soot particles in methylene dichloride and separating the stripped particles in a syringe filter, as described above. The methylene dichloride was subsequently removed from the extract by evapoating at 50°C under a stream of nitrogen and the residue redissolved in DMSO.

Table 1. Measured amount of extract from soot particles collected 2.0 cm above burner.

Experiment number	Particle weight, mg	Extract weight, mg	% Extract
1	39.4	4.80	12.2
2	25.9	3.50	13.6
3	29.6	4.32	14.6

Table 2. Principal compounds identified in polycyclic aromatic hydrocarbons extracted from polycyclic aromatic hydrocarbon-coated soot particles (mass fraction).

Compound	Experiment 1	Experiment 2	Experiment 3	Method
Acenaphthylene	0.182	0.170	0.111	GC
Cyclopenta[*cd*]pyrene	0.108	0.182	0.085	LC
Pyrene	0.034	0.032	0.039	GC
Coronene	0.018	0.034	0.013	LC
Benzo[*ghi*]pyrene	0.017	0.029	0.019	LC
Naphthocoronene	0.019	0.026	0.009	LC
Anthanthrene	0.011	0.021	0.013	LC
Indeno(1,2,3-cd]pyrene	*0.019*	*0.011*	*0.013*	*LC*
Fluoranthene	0.017	0.016	ND	GC
Naphthalene	0.015	0.015	0.008	GC
Phenanthrene	0.012	0.011	ND	GC
Ovalene	0.011	0.013	0.007	LC
Benzo[*a*]pyrene	0.008	0.013	0.007	LC
Fluorene	0.006	0.005	ND	GC
Anthracene	0.003	0.003	ND	GC

Abbreviations: GC, gas chromatography; LC, liquid chromatography; ND, not detected.

Mutation Experiments

In the mutagenicity assay, 6×10^6 cells per 12-mL replicate culture were exposed to the substances to be tested. For the dose–response assays, the exposure times were 28 hr. In a separate series of experiments, cells were contacted with a fixed concentration of soot particles (extract-equivalent concentration of 0.1 μm/mL) for times ranging between 8 and 72 hr.

For dose–response tests, cultures with extract were prepared in concentrations of 1.0, 0.3, 0.1, 0.05, 0.02, and 0.01 μg extract/mL, by adding to the cultures the necessary amount of the extract dissolved in DMSO. The stock solution of extract in DMSO was first diluted so that the concentration of DMSO in the culture was kept at the desired level (0.05% by volume). The coated and stripped particles were added to cultures via the colloidal suspension of these particles in phosphate-buffered saline solution. The particle concentration of the stock solution was adjusted so that the volume of saline solution added to the culture would not exceed 1% by volume. In the series of experiments conducted with coated particles in the presence of DMSO, the necessary amount of DMSO was added to bring its concentration in the culture to 0.05% or 0.5% by volume. The particle concentrations used in the mutation assays were such that the concentration of the adsorbed PAH (in the case of coated particles) corresponded to 1.0, 0.3, 0.1, 0.05, 0.02, and 0.01 μg extract/mL, and in the case of stripped particles to the equivalent concentrations of extract if the particles had not been stripped.

Immediately after exposure, each cell culture was centrifuged and resuspended in 50 mL of fresh medium (in the cultures containing coated or uncoated particles these were observable in suspension and could not be completely removed in the centrifugation and resuspension process). One day later the cultures were counted and sufficient fresh medium was added to bring the cell concentration to 2×10^5 cells/mL. The cultures were then allowed to grow for 2 additional days without dilution. The 3-day growth period allowed expression of the trifluorothymidine-resistant mutant phenotype. Cultures were then plated in the presence and absence of trifluorothymidine in two 96-well microtiter plates, following the procedure described by Furth et al. (*16*). For the positive control 5 μg/mL of BaP was used. After a 13-day incubation period, the plates were scored for the presence of colonies in each well. The mutant fraction was determined by the frequency of trifluorothymidine-resistant cells in the exposed cell population. Data were analyzed according to the procedure of Penman and Crespi (*17*).

Results and Discussion

The extract exhibited strong mutagenic effects, as shown in Figure 1. Exposure to 0.05 μg/mL or less of the extract produced a clear positive response. The extract was also more toxic to the cells than the soot particles and the substrate (Fig. 2). Cells exposed to 1.0 μ g/mL of the extract did not resume growth within 3 days after exposure (survival less than 10%). The stripped particles, on the other hand, showed no evidence of inducing mutations up to the highest concentration tested (Fig. 1). The stripped particles were also nontoxic at all concentrations tested.

The results obtained with unextracted soot particles are also shown in Figure 1. These particles induced a statistically significant mutagenic response at concentrations above 0.1 μg/mL of extract. At concentrations of 1.0 μg/mL, the coated particles were also fairly toxic, killing about 50% of the cells. The mean mutant fraction for concentrations at and above 0.1 μg/mL was statistically significant when compared to both the historical and the concurrent negative control data. Although lower concentrations did not induce a statistically significant positive response, the data suggest a dose–response relationship. The mutagenicity of the soot particles was somewhat reduced by the addition of DMSO to the cultures, as is illustrated by the results shown in Figure 3. However, the PAH adsorbed on soot particles was clearly less mutagenic and less toxic than the same material in the form of an extract over the exposure time used in the *in-vitro* dose–response tests described.

The effect of time of exposure on mutagenic response induced by soot particles is shown in Figure 4. It is interesting to note that the observed response occurs after a distinct induction time. Under the conditions of the experiment, this result implies that a build-up of the mutagen concentration must occur before its effects become evident.

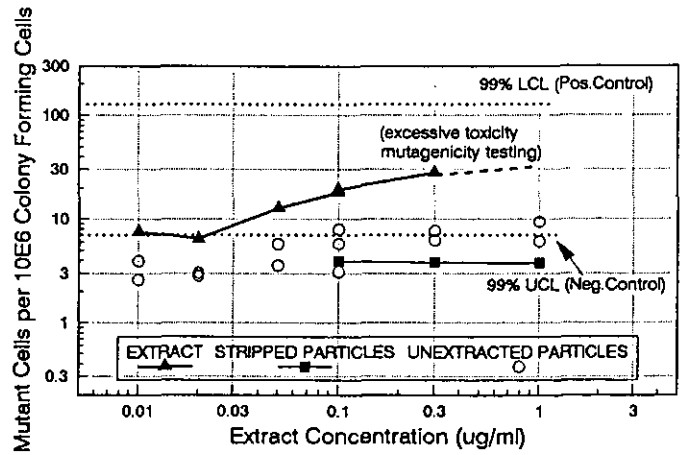

FIGURE 1. Mutagenicity of soot particles and extract. Tested with MCL-3 cells, 28-hr exposure time.

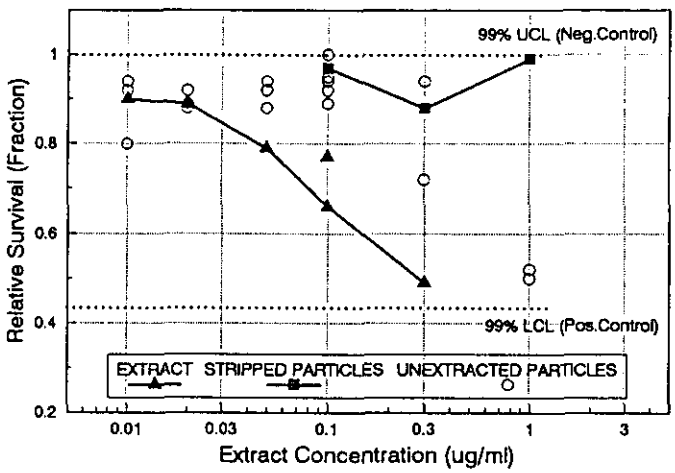

FIGURE 2. Toxicity of soot particles and extract. Tested with MCL-3 cells, 28-hr exposure time.

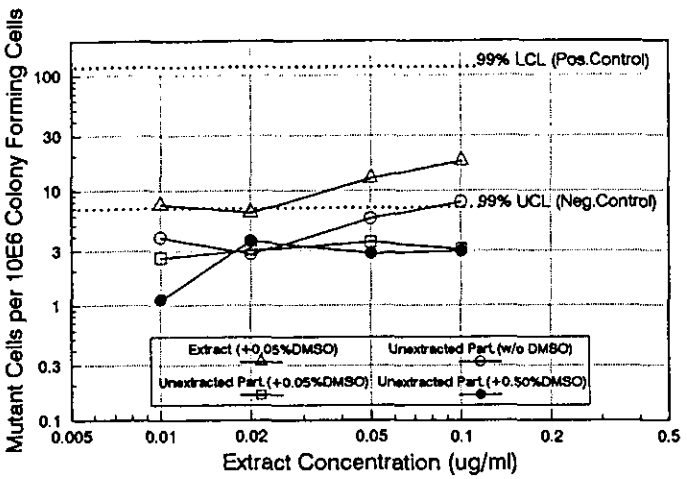

FIGURE 3. Mutagenicity of soot particles and extract with added dimethyl sulfoxide. Tested with MCL-3 cells, 28-hr exposure time.

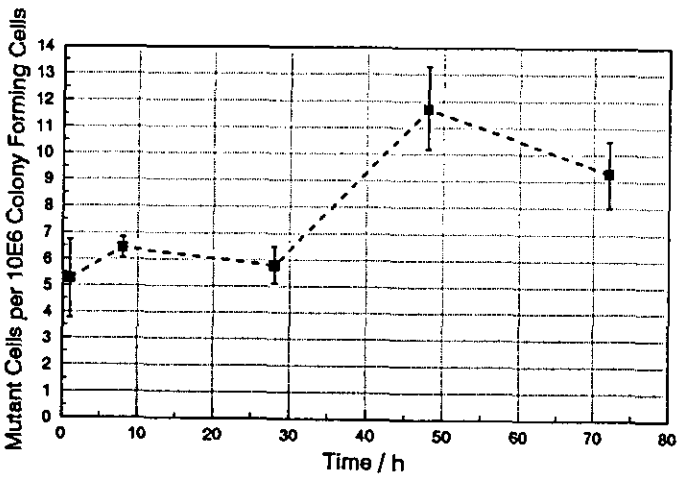

FIGURE 4. Mutagenicity of soot particles as a function of exposure time. Tested with MCL-3 cells, extract concentration 0.1 μg/mL.

The results on dose-response for the extract are in substantial agreement with those previously reported by Crespi et al. (*3*) for the mutation in metabolically competent AHH-1 cells when exposed to combustion-generated carbonaceous particles and a methylene dichloride extract of the same particulates. Although the reported mutagenicity induced by the extract was lower, which may be due to a lower sensitivity of the AHH-1 cell line or a different composition of the PAH mix, the relative mutagenicity of PAH in the form of extract and particle coating was approximately the same.

Bond et al. (*4*) have found, on the other hand, that retention times of mutagens inhaled in the form of adsorbates on respirable size particles in the lung are much longer than those of the same materials inhaled as droplets of the pure substances. These observations imply that bioavailability—the transfer of mutagenic material from particles to cells—is an essential issue for describing the activity of inhaled mutagens. The transfer of mutagens adsorbed on particle surfaces to cells can occur either by phagocytosis or by desorption, diffusion, and mass transfer across cell membranes.

In the present experiments, the ratio of soot particles (represented as 1 μm aggregates of 50 nm primary particles) to cells is estimated as 3000:1 at the highest concentration tested and 30:1 at the lowest, sufficient to ensure ample cell–particle contact in the culture. If phagocytosis were the dominant mechanism for the transfer of mutagens to the cell, an approximately linear relation between induced mutations and exposure time would be expected. The induction time observed in the time–response curve of Figure 4 suggests that mass transfer by desorption, diffusion, and transfer across the cell membrane is the prevalent mechanism. In this case the surface characteristies and morphology of the soot particles (primary particle size, morphology, metallic additives) could have a marked effect on the rate of release of adsorbed materials and hence on the overall mutagenicity of particular soot particles. Further research on this issue is in progress.

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