# Isolation and quantitative estimation of diesel exhaust and carbon black particles ingested by lung epithelial cells and alveolar macrophages in vitro

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*A new procedure for isolating and estimating ingested carbonaceous diesel exhaust particles (DEP) or carbon black (CB) particles by lung epithelial cells and macrophages is described. Cells were incubated with DEP or CB to examine cell-particle interaction and ingestion. After various incubation periods, the cells were separated from free extracellular DEP or CB particles by Ficoll density gradient centrifugation and dissolved in hot sodium dodecyl sulfate detergent. Insoluble DEP or CB residues were isolated by high-speed centrifugation, and the elemental carbon (EC) concentrations in the pellets were estimated by a thermal-optical-transmittance method (i.e., carbon analysis). From the EC concentration, the amount of ingested DEP or CB could be calculated. The described technique allowed the determination of the kinetics and dose dependence of DEP uptake by LA4 lung epithelial cells and MHS alveolar macrophages. Both cell types ingested DEP to a similar degree; however, the MHS macrophages took up significantly more CB than the epithelial cells. Cytochalasin D, an agent that blocks actin polymerization in the cells, inhibited approximately 80% of DEP uptake by both cell types, indicating that the process was actin-dependent in a manner similar to phagocytosis. This technique can be applied to examine the interactions between cells and particles containing EC and to study the modulation of particle uptake in diseased tissue.*

## INTRODUCTION

Diesel exhaust particles (DEP) from traditional diesel engines and fuels have been shown in animal and cell studies to cause a variety of health effects including pulmonary inflammation, increased severity of infections, and allergic lung disease (1–7). Earlier animal studies also suggested an association between DEP inhalation and elevated risk of thoracic cancer (8); however, more current reassessments of these studies, combined with new research, suggest that the cancers observed in the DEP-exposed rodents could result from a nonspecific particle-overload effect (9–11). A significant portion of inhaled DEP is ingested by alveolar macrophages and cleared from the lungs through the mucociliary escalator mechanism (12–14). The fate of DEP in lungs and their interactions with lung cells are less well understood. In vitro uptake of DEP by epithelial cells lining the bronchial airways has been reported (15,16), and electron microscopy evidence shows the presence of DEP in lung epithelial cells of guinea pigs exposed to diesel engine exhaust (17). Since human and rodent lung epithelial cells outnumber alveolar macrophages by a factor of 5 to 10 (18), any contribution of epithelial cells to inflammatory processes and particle clearance could be a significant factor in determining the health effects of DEP. However, there is limited quantitative evidence explaining the relative efficacies of macrophages and epithelial cell ingestion of DEP and other airborne particles. Such knowledge would help explain the mechanisms of particle-induced lung diseases, such as fibrosis and cancer, and could be of use in targeting therapies to particular cell types.

DEP are comprised of an elemental carbon core onto which thousands of different organic molecules are adsorbed. The carbon contents of airborne particles derived from the combustion of fossil fuel can therefore be separated into elemental carbon (EC) and organic carbon (OC) fractions. We hypothesized that determination of EC in biological tissues could serve as a measure of uptake of carbonaceous particles, such as DEP, because mammalian cells do not contain any EC per se. Here we describe a technique to isolate and chemically quantify microgram amounts of DEP from cells exposed to known amounts of particles. Using this method, the kinetics and dose-response characteristics of DEP uptake by murine epithelial and macrophages were examined. In addition, the uptake of pure carbon black (CB) versus diesel was compared to determine whether the presence of organics on the DEP influenced this process. Finally, to investigate whether particle ingestion by cells was a result of active cytoskeletal processes, the effect of the phagocytosis inhibitor cytochalasin D on DEP uptake was assessed.

## MATERIALS AND METHODS

### Cells and Reagents

LA4, a nontumorogenic murine lung epithelial cell line derived originally

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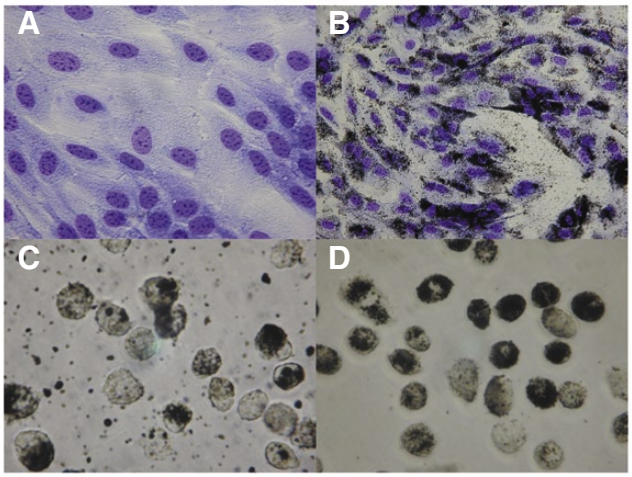


**Figure 1. Uptake of diesel exhaust particles (DEP) by LA4 mouse lung epithelial cells.** LA4 cells were cultured with 100 µg/mL DEP for 18 h. Cells were then isolated by trypsinization and purified by Ficoll density gradient centrifugation. (A) Appearance of control LA4 cultures after fixation and staining. (B) Appearance of DEP-treated LA4 cells after fixation and staining. (C) Appearance of DEP-treated LA4 cell suspension after isolation by trypsinization. Note accumulation of DEP inside some LA4 cells and a large number of free diesel exhaust particles outside the cells. (D) Appearance of DEP-treated LA4 cell suspensions after isolation by trypsinization and purification by Ficoll density gradient centrifugation. Note the absence of free DEP outside the cells.

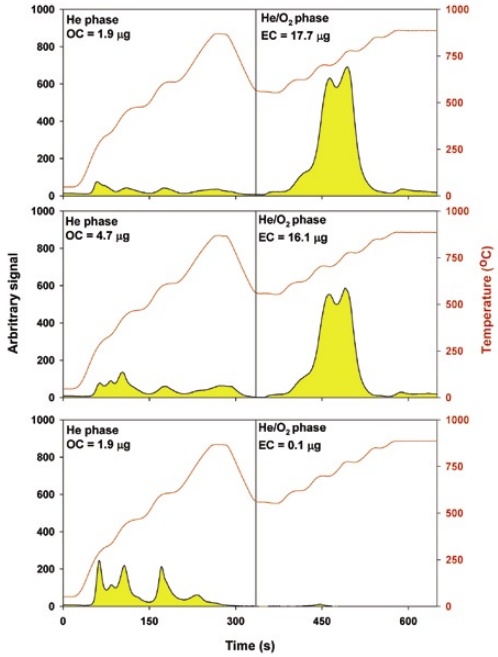


**Figure 2. Analysis of elemental carbon (EC) and organic carbon (OC) in various samples.** In each panel, the left side of the thermogram from 0 to 330 s constitutes the heating phase in helium atmosphere, and carbon peaks in this area denote organic carbon. Time point when the gas phase was shifted from helium to a mixture of helium and oxygen is denoted by the vertical line in the center of each thermogram. Carbon peaks in this second heating phase represent elemental form of carbon. (A) Analysis of National Institute of Standards and Technology diesel exhaust particles (NIST-DEP) preparation used in this study. (B) A typical analysis of a DEP/silica pellet isolated from DEP-exposed cells. (C) The results of the analysis of a silica pellet obtained from the processing of $2 \times 10^6$ control LA4 cells without prior exposure to DEP. Amounts of OC and EC in helium and $He/O_2$ phases of all thermograms are indicated within the figure.

from a urethane-induced epithelial adenoma in A/J mice (19), and the MHS cell line, derived from simian virus 40 (SV40) transformation of mouse alveolar macrophages (20), were obtained from ATCC (Manassas, VA, USA). Both cell lines were maintained in RPMI-1640 culture medium supplemented with 2 mM glutamine, 25 nM HEPES buffer, 20 µg/mL gentamycin, and 10% (v/v) fetal bovine serum. DEP (DEP Standard Reference Material 2975, average particle diameter 120 nm, surface area 108 $m^2/g$) were purchased from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). Fine CB particles (CB, Printex 90, average particle diameter 12 nm, surface area 300 $m^2/g$) were a gift from Dr. Vicki Stone, Napier University, Edinburgh, Scotland. Lympholyte-M, a Ficoll suspension used for isolating viable mouse cells by density gradient centrifugation was obtained from Accurate Chemical and Scientific (Westbury, NY, USA).

### In Vitro Exposure of Cells to DEP and Ficoll Density Gradient Separation

A DEP/CB stock suspension (5 mg/mL) was prepared in sterile normal saline and sonicated for 1 min using a probe sonicator (Microson Ultrasonic Cell Disrupter; Misonix, Inc., Farmingdale, NY, USA) at maximum amplitude. Cells were seeded in culture at a cell density of $2 \times 10^4$/mL in 6-well culture plates (5 mL cell suspension/well), and after 72 h, the sonicated DEP/CB suspension was added at desired concentrations. Control and DEP/CB-treated cells were harvested at the desired time intervals by trypsinization [trypsin-EDTA 1× from Gibco (Invitrogen, Carlsbad, CA, USA); 2 min] and collected by centrifugation at 150× *g* for 10 min. Cell pellets were resuspended in 5 mL RPMI medium containing 1% fetal calf serum (FCS), layered on top of 2 mL lympholyte-M, and centrifuged at room temperature at 280× *g* for 20 min. Cells with ingested DEP formed a black band at the Ficoll-RPMI interface, whereas free DEP or CB and any dead cells settled at the bottom of the Ficoll layer. Cells harvested from the interface were washed twice with RPMI medium containing 1% (v/v) FCS and counted in a hemocytometer. Viability of the cells at this stage was >95%.

### Isolation of DEP Taken Up by Cells

A 2% sodium dodecyl sulfate (SDS) solution (0.5 mL; 2% w/v in normal saline) was added to 0.5 mL Ficoll-purified cell suspension containing a known numbers of cells, and the mixture was vortex mixed immediately. Tubes of cells in SDS were kept in 100°C boiling water for 15 min with intermittent vortex mixing to completely solubilize the cellular constituents. Ingested DEP/CB remained insoluble in SDS and were isolated by high-speed centrifugation in an Eppendorf microfuge at 13,000× *g* for 20 min. In order to increase the bulk of the DEP/CB pellet and prevent their loss during washing steps, 100 µL 5 mg/mL suspension silica powder (Sigma-Aldrich, St. Louis, MO, USA) in normal saline were added to each tube at the same time that SDS was added. DEP/CB/silica pellets were washed once with 1 mL hot 1% SDS and twice with 1 mL normal saline, suspended in 50 µ L normal saline, and transferred to 1.5-$cm^2$ quartz filter punches. Filters were dried overnight under vacuum at 50°C and analyzed using a carbon analyzer.

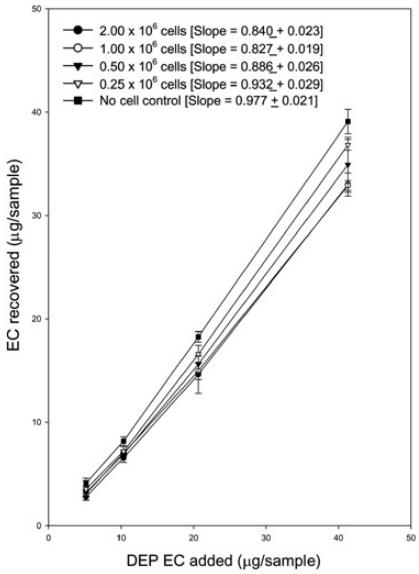


**Figure 3. Effect of cell numbers on diesel exhaust particles (DEP) recovery.** LA4 cells (2.00, 1.00, 0.50, 0.25, and 0 × $10^6$/tube) were mixed with different given amounts of DEP, and the samples were processed for DEP estimation. Elemental carbon (EC) recovered from each sample was estimated by EC/organic carbon (OC) analysis on a carbon analyzer. Each point represents mean ± SD of four replicate and independent EC determinations.

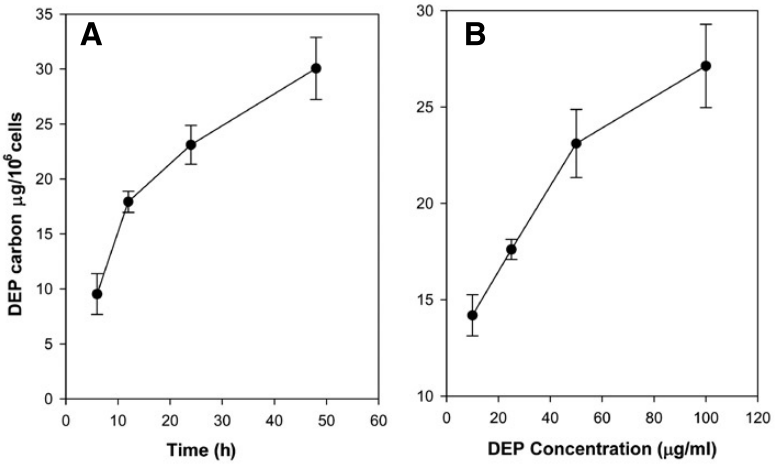


**Figure 4. Kinetics and dose dependence of diesel exhaust particles (DEP) uptake by LA4 cells in culture.** (A) LA4 cells were cultured in 6-well culture plates in the presence of 50 µg/mL DEP for 6, 12, 24, and 48 h. Cells were isolated and purified on a Ficoll density gradient and subjected to sodium dodecyl sulfate (SDS) treatment in the presence of silica particles. Elemental carbon (EC) in the DEP/silica pellets was determined on a carbon analyzer. Each mean ± SD value represents data from four independent culture wells. (B) LA4 cells were cultured in 6-well culture plates with 10, 25, 50, and 100 µg/mL DEP for 24 h, and DEP uptake was estimated as described for panel A.

### Carbon Analysis

The OC and EC in the DEP/CB/silica pellets were measured using thermal-optical-transmittance (TOT; Sunset Laboratories, Forest Grove, OR, USA) and the National Institute for Occupational Safety and Health Method 5040 for estimating carbon in diesel particulate matter (21). Exhaustive details of the method have been provided elsewhere (22,23). Briefly, vacuum-dried DEP/CB/silica samples on quartz filters were heated stepwise to ~850°C in a temperature-programmable oven under helium (He phase). Organic matter volatilized under these conditions was oxidized to $CO_2$ in the presence of a $MnO_2$ catalyst and then reduced to methane using a Ni metal hydride catalyst. The methane generated was detected and quantified by a flame ionization detector (FID), and the OC in the sample was computed from the quantity of methane evolving during this step. To analyze EC contents, after a brief cooling respite, the samples were again heated stepwise to ~900°C in a helium-oxygen environment, whereby the nonvolatile EC was oxidized to $CO_2$. This $CO_2$ was then converted to methane, and the EC was quantified as before. EC ingested per million cells could be calculated from the values of EC in samples and the number of cells used for isolating the EC samples.

### Statistical Analysis

Linear regression analyses were performed using Sigmaplot 8.0 software (SSI, San Jose, CA, USA).

## RESULTS

### Uptake of DEP by LA4 Murine Lung Epithelial Cells

LA4 epithelial cells were incubated with DEP, washed, and stained for microscopic examination. Figure 1 shows the control (panel A) and DEP-exposed (panel B) LA4 cells after 18 h culture with DEP or vehicle solution. Figure 1B shows a significant accumulation of DEP in the LA4 cells that could be due to ingestion of DEP and/or possibly to adherence of DEP to LA4 cells. When LA4 cells were incubated with DEP at 4°C, no association of DEP particles with cells was seen (results not shown), suggesting that the DEP accumulation could be due to an energy-dependent ingestion process. DEP-exposed LA4 cells were detached by trypsinization and vigorously agitated using a pasture pipet in order to detach loosely adhered DEP from the LA4 cells. At this stage, the suspension contained LA4 cells with ingested DEP as well as considerable amounts of extracellular DEP in free suspension (Figure 1C). Figure 1D shows LA4 cells free from extracellular DEP after purification by Ficoll density gradient centrifugation. It should however be emphasized that only agglomerates of DEP/CB particles may be detected by light microscopy. Adherence of superfine particles invisible by light microscopy to the membranes of the Ficoll-purified cells cannot be ruled out from our results. Ficoll density gradient-purified cells containing DEP were solubilized in hot 1% SDS, and the ingested DEP that remained undissolved were isolated by high-speed centrifugation in the presence of silica powder, as described in the Materials and Methods section.

Figure 2A shows a typical TOT thermogram obtained for the standard NIST-DEP preparation. The initial helium phase shows several small peaks that account for 1.9 µg OC. The He/$O_2$ phase shows multiple co-evolving peaks that account for 17.7 µg EC. EC accounted for about 90% of the total carbon in the NIST-DEP. It should be noted that DEP

contain some noncarbonaceous materials as well as moisture, which would decrease the actual percentage by weight of EC in DEP. Figure 2B shows a TOT thermogram of the DEP/silica pellet isolated from DEP-exposed LA4 cells. The OC and EC values were 4.7 and 16.2 µg, respectively. The greater proportion of OC in this case was likely due to the SDS that may have adsorbed onto DEP/silica particles during the process of isolating ingested DEP. The thermogram in Figure 2C for the control silica pellet (i.e., processed LA4 cells not exposed to DEP) showed no evidence of an EC contribution from cells or SDS.

### DEP Recovery from Cells and Sensitivity of DEP Detection

The procedure for isolation of DEP ingested by cells involved treatment with hot SDS solution and several wash steps. Some loss of DEP could be expected during this processing. To assess recovery of DEP during sample processing, we spiked LA4 cells in suspension with 4, 10, 20, or 50 µg DEP and processed the samples through the SDS, silica, and wash steps. Figure 3 shows the relationship between the TOT-measured EC in DEP mixed with different numbers of LA4 cells (0, 0.25, 0.50, 1.00, and 2.00 $\times 10^6$ cells, no Ficoll density treatment). Regression coefficients ($R^2$) for all five plots in Figure 4 were >0.99, indicating a close to perfect correlation for each curve. Slopes of the linear regression lines indicated the recovery of DEP after the SDS treatment and washings. When DEP was processed in the absence of LA4 cells, the slope of the regression line was 0.977 ± 0.021, indicating that 97.7% of the DEP EC was recovered. However, when DEP was mixed with 0.25, 0.50, 1.00, and 2.00 million LA4 cells, the slopes of the regression lines were 0.932, 0.885, 0.827, and 0.840, respectively; thus, the recoveries of DEP declined as cell concentration increased. Because of this potential loss, all subsequent uptake experiments were conducted using 0.25 million cells, which would yield a recovery of >90% of the ingested DEP.

The average standard deviation for mean values of EC estimations at the lowest dose of DEP in Figure 3 was 0.32 µg. If the estimated quantification limit is defined as three times the standard deviation units above zero, the lower limit of detection of DEP EC by this procedure is approximately 1 µg. Since EC is 68% by weight of our DEP preparation, sensitivity of estimating DEP uptake would be approximately 1.5 µg.

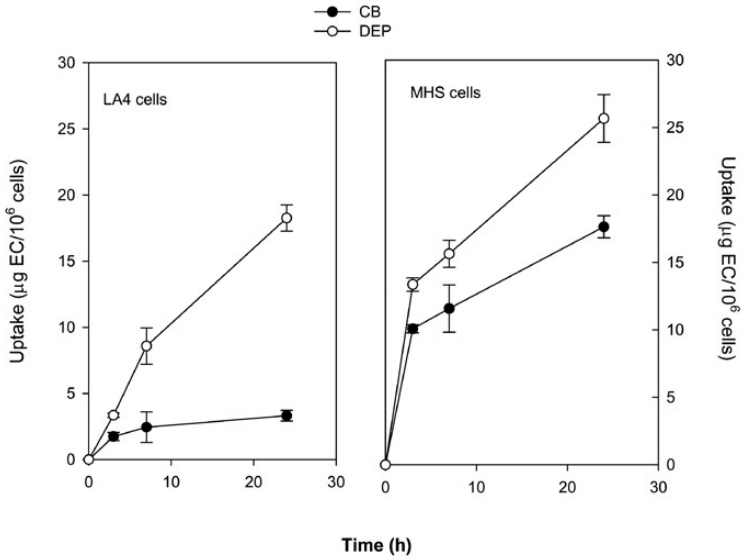


**Figure 5. Comparison of kinetics of uptake of diesel exhaust particles (DEP) and carbon black (CB) by LA4 and MHS cells.** LA4 and MHS cells in 6-well plates were incubated for 3, 7, and 24 h in the presence of 50 µg/mL DEP or CB. At each time point, cells were harvested and processed for the determination of ingested EC. Each data point represents mean ± SD of EC determinations from three independent assay wells.

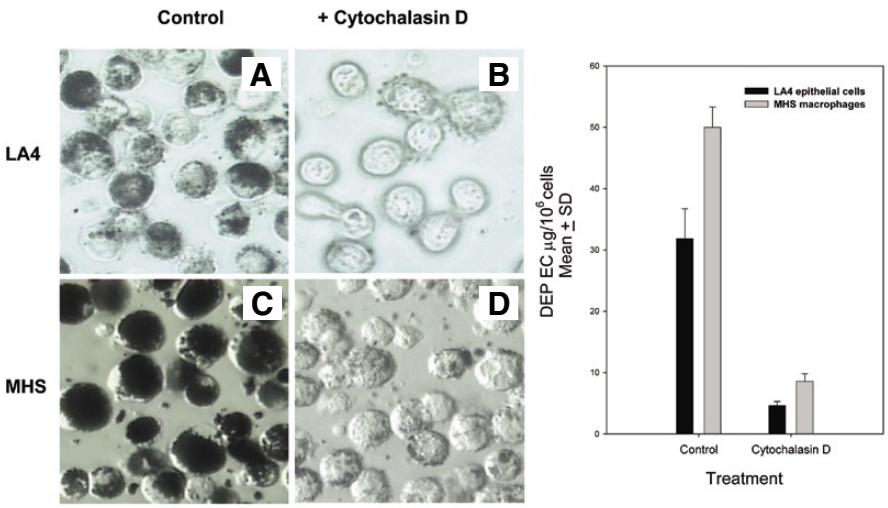


**Figure 6. Effect of cytochalasin D on diesel exhaust particles (DEP) uptake by LA4 and MHS cells.** LA4 and MHS cells were cultured in 6-well culture plates in the presence or absence of 2.5 µg/mL cytochalasin D for 1 h. DEP (100 µg/mL) were added, and incubation continued for an additional 24 h. At the end of the incubation, cells were photographed (left panel) and processed for determining the amount of DEP elemental carbon (EC) taken up by the cells. All values are mean ± SD of four independent estimations.

### Time Kinetics and Dose Response of DEP Uptake by LA4 Cells

Using the procedure detailed above, we proceeded to study (*i*) the time kinetics and (*ii*) the dose dependence of DEP uptake by LA4 cells. Figure 4A shows the kinetics of particle uptake by LA4 cells exposed to 250 µg DEP in 5 mL (50 µg/mL) total culture medium. This dose corresponds to 1 ng DEP/cell in culture. By 6 h culture, approximately 10 µg DEP EC/$10^6$ cells were ingested. Uptake increased at later time points and was approximately 30 µg/$10^6$ cells at the 48-h time point. Figure 4B shows the effect of DEP concentration in the culture medium on the DEP uptake by LA4 cells over

24 h. At the highest dose of DEP (100 µg/mL), DEP uptake was approximately 25 µg/$10^6$ LA4 cells. Average uptake of DEP per cell can be computed from these results, although actual variations in DEP uptake by individual cells cannot be assessed by this technique. Our results indicate that LA4 lung epithelial cells can take up significant amounts of DEP, and this uptake is both time- and dose-dependent. Preliminary studies have since shown comparable uptake of DEP using a human alveolar epithelial cell line A549 and primary human airway epithelial cells (results not shown).

### Kinetics of Uptake of DEP and CB by LA4 Cells and MHS Alveolar Macrophage Cell Line

The kinetics of uptake of DEP and CB by epithelial cells and macrophages was compared. The results in Figure 5 indicate that the rate of DEP uptake by LA4 cells was significantly greater than the uptake of CB. At 24 h, LA4 cells had ingested 18 µg DEP EC/$10^6$ cells, whereas the corresponding figure for CB was only 3.5 µg/$10^6$ cells. Uptake of DEP by MHS cells on a per million cell basis was only 30% greater than LA4 cells, whereas the uptake of CB EC was markedly (5-fold) greater in MHS cells.

### Effects of Cytochalasin D on DEP Uptake by Epithelial Cells and Macrophages

In order to gain insight into the mechanism of DEP uptake by the two cell types, the effect of the actin polymerization inhibitor cytochalasin D was examined. Photomicrographs in Figure 6 show that, compared with the control cells, DEP uptake by cytochalasin D-treated LA4 and MHS cells was much lower. Quantitative estimation indicated that cytochalasin D treatment resulted in an 80% decrease in DEP uptake by both cell types (Figure 6, right panel). Similar results were obtained by using CB (results not shown). These results suggest that both cell types take up DEP particles through an actin polymerization-dependent mechanism and further confirm that the particles isolated from LA4 and MHS cells were ingested and stored in intracellular compartments.

## DISCUSSION

The two main types of lung cells that encounter inhaled diesel particles are epithelial cells and alveolar macrophages. Macrophages are professional phagocytes and ingest inhaled particles through actin-dependent formation of phagosomes and other endocytic pathways (24–26). Epithelial cells are not commonly regarded as phagocytes, although electron microscopic analysis has revealed DEP particles inside the lung epithelial cells of guinea pigs exposed to diesel exhaust (17). To understand the relative contributions of macrophages and epithelial cells in removing DEP that reach lung alveoli, it was necessary to estimate the ability of these cells to ingest DEP. For this purpose, we developed a technique that could be used to isolate and measure microgram quantities of DEP and CB ingested by cultured cells. In principle, the procedure assumes that cells do not contain any EC of their own and that the EC estimated in insoluble residues after solubilizing the cells is entirely due to that of ingested particles. This assumption was indeed valid, as no EC was detected in control LA4 or MHS cells processed in the same way as the DEP-exposed test samples.

After incubation of LA4 and MHS cells with DEP, microscopic examination indicated the presence of significant amounts of black particles inside the cells. Mere adherence of the particles to the cell membranes appeared unlikely, since incubation at 4°C prevented any

accumulation of the particles in the cells. Moreover, cytochalasin D treatment that interferes with actin-myosin polymerization (27) also blocked the intracellular accumulation of DEP or CB, indicating that the particles were likely ingested by the cells. A Ficoll density gradient centrifugation step was used to separate extracellular DEP and dead cells, and live cells containing DEP into two different layers. Intracellular DEP was then liberated by dissolving the purified cells in hot 1% SDS detergent solution, leaving the insoluble DEP to be isolated by high-speed centrifugation. Addition of amorphous silica as carrier particles prevented loss of DEP due to adherence to walls of centrifuge tubes, without interfering with the carbon analysis. Despite these precautions, small amounts of DEP were still lost presumably by binding to cellular components solubilized by SDS. Quantitatively, this loss depended upon the number of cells and could be as high as 15% if 2 million LA4 cells were used per sample. The cell culture studies were performed in 6-well plates, where cell recovery numbers approximated 0.5 million and the loss of DEP remained below 10%.

The EC in DEP or CB pellets could be quantitatively estimated using a carbon analyzer. The particulate matter uptake results were presented in units of micrograms of DEP or CB EC per million cells. Since the EC was entirely due to the carbonaceous particles however, absolute amounts of ingested DEP/CB per million live cells could also be computed by knowing the percentage by weight of EC in DEP (68.8%) and CB (82.5%). Analysis of several concentrations of the particles in the culture medium showed that the maximum accumulation of DEP EC in LA4 cells was approximately 30 $\mu g/10^6$ cells, which would correspond to about 45 µg actual DEP/$10^6$ cells. Kinetic studies comparing macrophages and epithelial cells indicated the initial rate of accumulation of DEP EC was far greater in macrophages (>3 $\mu g/10^6$ cells/h during the first 3 h) than in the LA4 cells (1 $\mu g/10^6$ cells/h). Overall however, accumulation of DEP EC at the later time point (24 h) was comparable (18 and 23 $\mu g/10^6$ cells in LA4 and MHS cells, respectively), although it should be kept in mind that LA4 cells (diameter of detached rounded up cells 15–20 µm) have 2- to 3-fold greater volume than MHS cells (diameter 10–15 µm). This would suggest that the MHS cells take up more DEP on a per unit cell volume basis. Interestingly, the uptake of CB by LA4 cells was <20% of the DEP uptake, indicating that these cells have a significantly greater affinity for DEP. This difference was less marked for the MHS macrophages, since CB uptake was 70% that of the DEP. These results suggest that the rate of uptake of particulate material by lung epithelial cells may be more sensitive to the physicochemical properties of the particle (that are significantly different for DEP and CB; see Reference 28) as compared with MHS cells, because differences in the relative uptake of DEP and CB were greater for LA4 than in MHS cells.

In summary, we have developed a new technique that can be used to isolate and estimate minute amounts of carbonaceous particles ingested by cells in culture. Using this method, we were able to compare the kinetics of DEP/CB uptake by lung epithelial cells and alveolar macrophages. Since the method is based upon estimating the amount of EC in the particles isolated from cells, it can in principle be used to measure uptake of other kinds of particles containing EC. We have recently studied the uptake of carbon nanotubes and nanodiamonds by lung epithelial cells and have modified the technique to assess the deposits of EC in lungs of healthy human subjects as well as in human smokers and coal miners (Saxena et al., manuscript under preparation). We expect that this new technique will enable further objective studies of the interactions between different types of carbonaceous particles and lung cells.

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## COMPETING INTERESTS STATEMENT

*The authors declare no competing interests.*

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