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Suppression of Alveolar Macrophage Membrane-Receptor-Mediated Phagocytosis by Model Particle-Adsorbate Complexes: Physicochemical Moderators of Uptake

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In order to assess the abilities of alveolar macrophages (AMs) to phagocytize adsorbent-adsorbate complexes, rat AMs were incubated *in vitro* with two carbon blacks that have 15-fold differences in specific surface areas (ASTM classification N339 < Black Pearls 2000) sorbed with 0.5 and 1.0 monolayer coverages of a polar and semi-polar adsorbate (acrolein and benzofuran, respectively). One-half monolayer coverages of N339 with either adsorbates significantly suppressed the phagocytosis of the carbon black, whereas one monolayer coverage did not. Neither adsorbate at either coverages affected the phagocytosis of Black Pearls 2000. The capacity of macrophages to phagocytize a subsequent particle challenge via the Fc-membrane receptor was quantified following treatment of the macrophages with the carbon black-adsorbate complexes. Treatment of the macrophages with carbon black N339-adsorbates complexes at both coverages impaired Fc-receptor-mediated phagocytosis, whereas no effect was observed when the carbon black was Black Pearls 2000. The results of this study indicate that the surface properties of the particles, the chemical properties of the chemical pollutants, and the interactions between particles and pollutants play a major role in defining the biological effect of particle-pollutant complexes.

Introduction

Pollutant gases or vapors that normally do not reach the distal regions of the respiratory tract may do so when sorbed on the surfaces of inhalable environmental particles. In combination, the particle and the adsorbed pollutant molecules may produce physiological effects not induced by either agent alone (*1,2*).

The first line of defense against inhaled particles in the distal lung is the alveolar macrophage phagocytic system (*3*). Particles reaching the alveolar regions are rapidly ingested by the macrophages, thereby effectively sequestering the deposited material from the vulnerable respiratory surface (*4*). Since the alveolar macrophage plays the pivotal phagocytic role in the lungs, the effect of *in vitro* treatment of the phagocyte with particle-adsorbate combinations has been tested.

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Carbon black alone is not an environmental hazard and is considered a nuisance dust because after exposure to environmentally relevant doses the particles do not appear to produce profound toxic effects in the lung parenchyma (*5-7*). Pollutant molecules sorbed on the surface of carbon black particles may alter the biologic response to the inhaled carbon black. Indeed, the previous paper in this series (*8*) reported that short-term treatment of alveolar macrophages with carbon black-adsorbate complexes suppressed Fc-receptor-mediated phagocytosis in a dose-related manner that was dependent on the adsorbate coverage of the carbon black.

In our previous study (*8*), the short-term treatment of the macrophages with the carbon black-adsorbate complexes did not result in their internalization by the phagocyte. Herein, we have extended the treatment of the macrophages with the carbon black and carbon black-adsorbate complexes to determine whether the presence, identity (i.e., chemistry), and coverage of the adsorbate altered the phagocytic ingestion of the carbon black particles. This study was to determine the effect of such treatment on the capacity of the macrophages to phagocytize a secondary particle challenge via its Fc-membrane receptor.

Materials and Methods

Chemicals and Sample Preparation

The adsorbates studied were acrolein and benzofuran (>99+% purity) (Aldrich Chemical Co.). Two amorphous oil furnace carbon black adsorbents were used: ASTM classification N339 and Black Pearls 2000 (BP 2000, Cabot Corporation).

The adsorbents were coated with two different coverages of adsorbates (0.5 and 1.0 equivalent monolayer). One monolayer is considered to occur when the adsorbent surface is covered by a unimolecular layer of adsorbate molecules. This concentration is a function of the specific surface area of the adsorbent and the cross-sectional area of the adsorbate molecule. Two methods of preparing the adsorbate-adsorbent complexes were used: *a*) A known amount of adsorbent was placed in a short (5–10 cm), wide bore (4 mm i.d.) stainless-steel gas chromatographic column, and the requisite amount of adsorbate was injected onto this column via a heated injection port. The column was maintained at room temperature, and the carrier gas (helium: 20 mL/min) carried the adsorbate onto the column (*10,11*). The column effluent was connected to a flame ionization detector to confirm that the adsorbate did not elute from the column. After about 10 min of equilibration, the column was removed from the gas chromatograph, and the coated adsorbent was unpacked from the column. *b*) A known amount of adsorbent was placed in a beaker, and the requisite amount of adsorbate was added in a sufficient amount of volatile solvent (dichloromethane) to completely cover the carbon black. The resulting suspension was stirred and the volatile solvent was allowed to evaporate completely. Known amounts of coated adsorbents were used to prepare the test suspensions. Preliminary experiments showed that the method of preparation had no effect on carbon uptake or the biologic effect of the carbon-adsorbate complexes.

All samples were prepared in RPMI tissue culture medium supplemented with 10% calf serum and containing 100 units of penicillin and 100 μg of streptomycin (RPMI medium). The adsorbents were tested at a concentration of 0.04 mg/mL. Preliminary experiments had determined that this concentration of the carbon black alone did not effect Fc-membrane receptor-mediated phagocytosis. All samples were sonicated for 30 min before addition to the macrophage monolayers to uniformly disperse the particles within the media and eliminate agglomeration.

Alveolar Macrophage Phagocytosis of Carbon Blacks

The lungs of 150 to 200 g Wistar rats (Hilltop Laboratory Animals, Scottdale, PA) were lavaged by previously described methods (*8*), and the cells were suspended in RPMI medium at a concentration of 5×10^5 cells/mL. A 0.2 mL aliquot of this suspension was then placed on 22-mm^2 coverslips in 35 × 10 mm plastic petri dishes and incubated at 37° C for 45 min in a 5% CO_2 incubator. After monolayering, the fluid was removed and immediately replaced with 1.5 mL of the carbon-adsorbate suspension. Following incubation at 37° C for varying times, the nonphagocytized carbon black-adsorbate suspensions were removed, the monolayers washed three times with warmed RPMI medium, and cell viability determined by exclusion of trypan blue. The monolayers were then dried, fixed with methanol, and stained with Wright-Giemsa. Alveolar macrophage cell density between untreated and treated monolayers was determined microscopically using a grid reticule in the microscope eyepiece. No significant differences were found in the cell density of macrophages between control and treated monolayers.

The dose of carbon black and carbon black-adsorbate complexes ingested by alveolar macrophages was quantified by image analysis (Dapple System). One hundred individual macrophages were visualized on the monitor at 1000× magnification. The image analyzer was set to record areas of defined blackness contrast. The amount of carbon black ingested was determined by measuring the unit area of carbon black particle blackness per macrophage. The data are expressed as the mean area (square micrometers) of carbon black ingested per 100 macrophages.

Alveolar Macrophage Fc-Membrane Receptor-Mediated Phagocytosis

Following incubation of the macrophages with the carbon black and carbon black-adsorbate complexes, Fc-membrane receptor-mediated phagocytosis was assayed (*9*) by adding 1.5 mL of 0.5% suspension of sensitized sheep erythrocytes (RBCs) in RPMI medium and incubating the resulting suspension at 37° C for 45 min. Noningested RBCs were hypotonically lysed by adding distilled water to the monolayers for 10 sec followed by several rinses with RPMI medium. The monolayers were then dried, fixed with methanol, and stained with Wright-Giemsa. The stained cell monolayers were read microscopically at 1000× to quantify the percentage of macrophages containing RBCs. In addition, the number of RBCs ingested per actively phagocytic macrophage was determined. The phagocytic index (total number of RBCs ingested by 100 macrophages) was calculated by multiplying the percentage of phagocytic macrophages by the mean number of RBCs ingested per phagocytic macrophage. One hundred macrophages were counted on each monolayer. The observed decreases in the phagocytic index are due to both a decrease in the percentage of phagocytic macrophages and a decrease in the number of RBCs ingested per phagocytic macrophage.

Statistical Analysis

For each carbon and carbon-adsorbate combination, two to three separate experiments were performed with three replicates within each experiment. Comparisons of the raw data between controls and treated groups were performed by the Student's *t*-test. All statements of significance are $p < 0.05$.

Results

It was critical that macrophage viability not be strongly affected by the samples assayed. Therefore, the viability of the cells was determined after the initial monolayering (> 95%) and after treatment with the samples (>90%). The only time that viability was less than 90% was after 18 hr of treatment with any given sample.

The effect of incubation time on carbon black ingestion is presented in Figure 1. More than 95% of the macrophages contained carbon black at the 2 hr assay period. With time, each of the phagocytic macrophages internalized more carbon black. The identity of the carbon black had no effect on particle ingestion. Figure 2 depicts a photomicrograph of macrophages that have ingested carbon black.

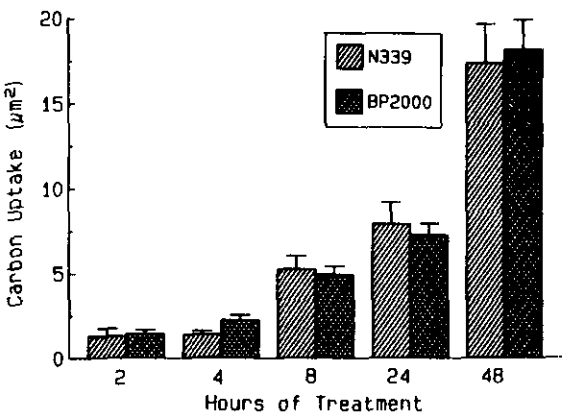

FIGURE 1. Comparison of alveolar macrophage phagocytosis of carbon blacks N339 and BP 2000. Each value represents the mean ± SE of six determinations.

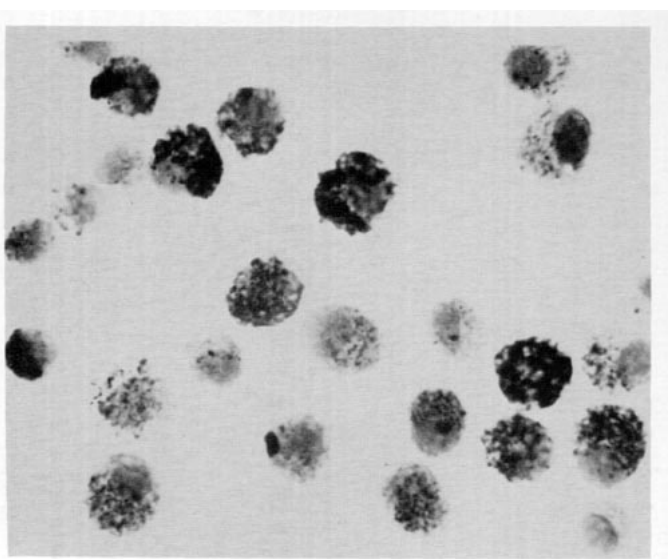

FIGURE 2. Photomicrograph of alveolar macrophages treated with carbon black BP 2000 for 24 hr.

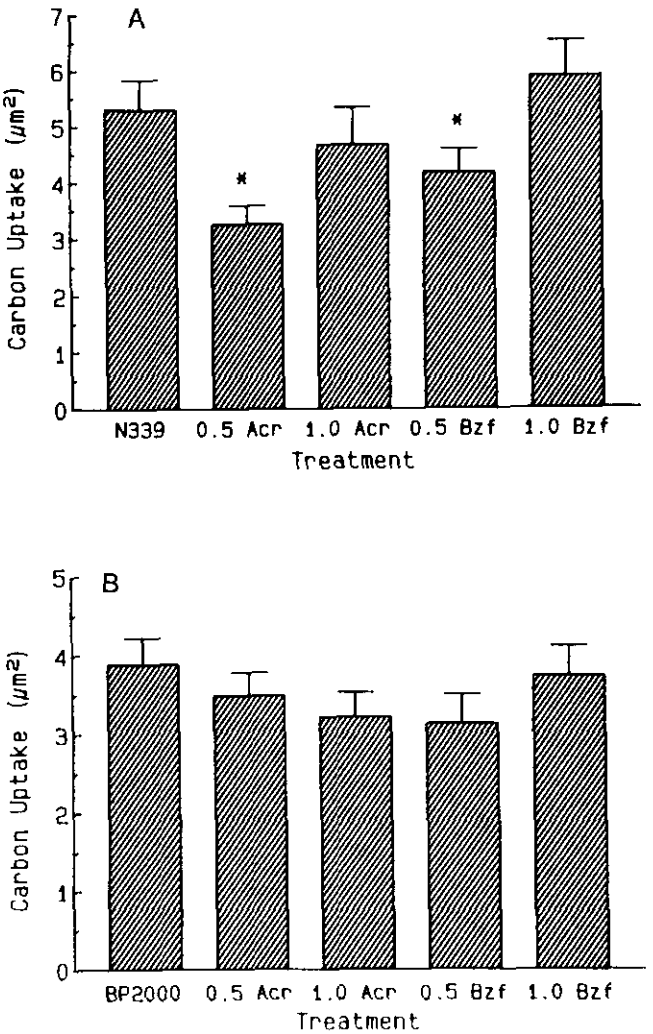

FIGURE 3. Comparison of the effect of 0.5 and 1.0 monolayer coverage with adsorbates (Acr, acrolein; Bzf, benzofuran) on the phagocytosis of carbon blacks N339 (*A*) and BP 2000 (*B*). Each value represents the mean ± SE of eight determinations; (*) $p<0.05$.

To determine the effect of adsorbates on carbon black particle phagocytosis, the carbon blacks, N339 and BP 2000, were sorbed with 0.5 and 1.0 monolayers of acrolein and benzofuran, respectively, and incubated with alveolar macrophages for 8 hr. Figure 3*A* shows that 0.5 monolayers of both acrolein and benzofuran significantly impaired the ingestion of carbon black N339, whereas 1.0 monolayers of the adsorbates did not. Neither 0.5 nor 1.0 monolayers of either of the adsorbates sorbed on BP 2000 had any effect on carbon black phagocytosis (Fig. 3*B*).

To determine the effect of carbon black and carbon black-adsorbate ingestion on the phagocytosis of a secondary particle, the macrophages were treated with the N339- and BP 2000-adsorbate mixtures for 8 hr followed immediately by Fc-receptor-mediated phagocytosis. Approximately 94% of the untreated control macrophages were phagocytic with each phagocytic macrophage ingesting approximately six RBCs. Preliminary studies had shown that treatment of the

macrophages with either N339 or BP 2000 without adsorbate at a concentration of 0.04 mg/mL had no significant effect on Fc-receptor-mediated phagocytosis (*8*). Figure 4*A* shows that both acrolein and benzofuran sorbed on carbon black N339 significantly suppressed Fc-receptor-mediated phagocytosis. Additionally, these suppressions were adsorbate dependent. In contrast, acrolein or benzofuran when sorbed on the carbon black BP 2000 had no significant effect on Fc-receptor-mediated phagocytosis (Fig. 4*B*).

Having demonstrated that treatment of macrophages with the N339-acrolein complex for 8 hr suppresses Fc-receptor-mediated phagocytosis, we next examined the longer term effect of carbon black-adsorbate ingestion on secondary particle phagocytosis. The macrophages were treated for 8 hr with the carbon black N339 sorbed with 0.75 monolayers of acrolein, the noningested particle-adsorbate complex was removed, the cells were replenished with fresh RPMI medium and incubated at 37°C. The coverage of 0.75 monolayers was used to increase dose while maintaining a coverage of less than one monolayer. Fc-receptor-mediated phagocytosis was performed at 4 hr and 12 hr thereafter. As shown before in Figure 4*A*, incubation of macrophages for 8 hr with acrolein sorbed on carbon black N339 significantly suppressed Fc-receptor-mediated phagocytosis (Fig. 5). After removal of the extracellular carbon black-adsorbate complex from the macrophage monolayer, an additional 4 hr of incubation in fresh media did not significantly alter macrophage phagocytosis (Fig. 5). Twelve hours of incubation with fresh media significantly enhanced the phagocytic capacity of untreated macrophages (Fig. 5), and treatment with carbon black N339 had no effect on this increase in the phagocytic index. In contrast, however, the macrophages treated with carbon black N339 sorbed with 0.75 monolayer of acrolein did not exhibit the phagocytic increase.

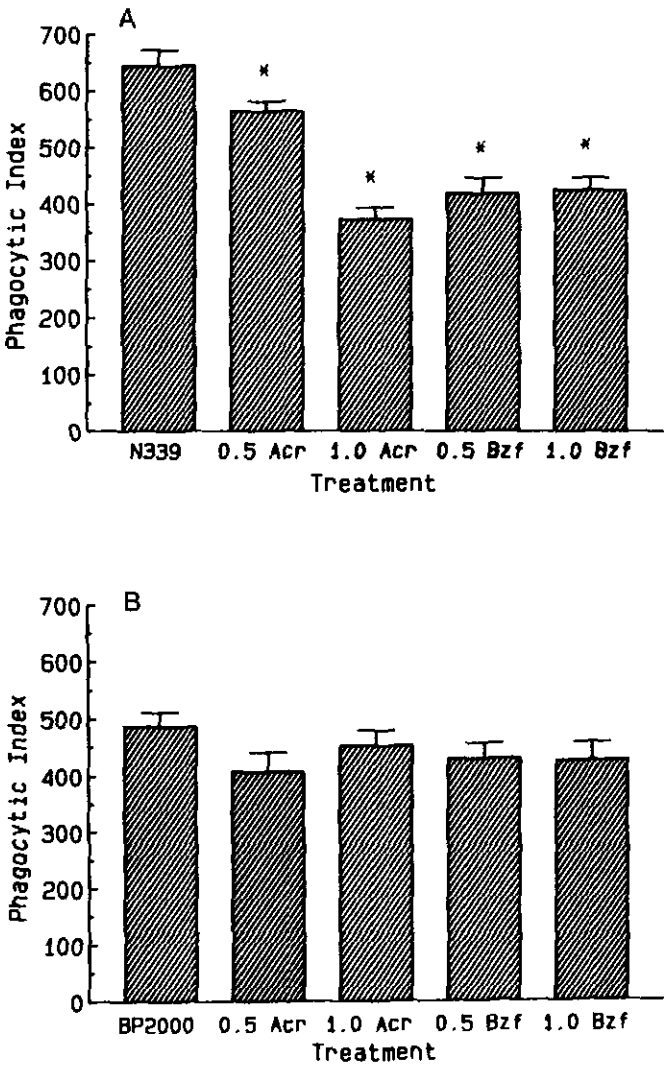

FIGURE 4. Comparison of the phagocytosis of a secondary particle via the Fc-membrane receptor following the treatment of the alveolar macrophages for 8 hr with carbon blacks N339 (*A*) and BP 2000 (*B*) at 0.5 and 1.0 monolayer coverages with adsorbates (Acr, acrolein; Bzf, benzofuran). Each value represents the mean ± SE of eight determinations; (*)$p<0.05$.

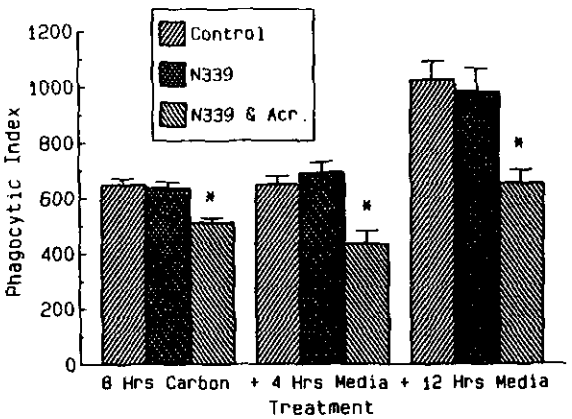

FIGURE 5. Comparison of Fc-membrane receptor-mediated phagocytosis following treatment of the alveolar macrophages for 8 hr with carbon black N339 at 0.75 monolayer coverage of acrolein (Acr) followed by an additional and 12-hr incubation with media. Each value represents the mean ± SE of eight determinations; (*)$p<0.05$.

Discussion

The rationale for this study was to test the biologic effect of simple particle-adsorbate complexes that model some of the important physical and chemical parameters of more complex environmental particulate-adsorbate combinations in order to predict the biologic effects of actual environmental pollutants emitted during the incomplete combustion of organic materials (*10,11*). The carbon blacks (adsorbents) selected for testing vary 15-fold in their specific surface areas (N339 < BP 2000) (*12*). This parameter was chosen to test whether the absolute amount of the adsorbates influenced the biologic response. Acrolein and benzofuran were selected as adsorbates on the basis of their environmental relevance (acrolein has been found in cigarette smoke and furans have been found in diesel particulate matter), on the basis of their differing polarities (dipole moment of benzofuran = 0.79 and acrolein = 3.12 Debye), and solubilities in water (benzofuran is insoluble and acrolein is partially soluble).

Comparison between the phagocytosis of N339 and BP 2000 showed that the kinetics of particle uptake were nearly identical. The size of the carbon black aggregate is approximately 30 nm in diameter, and no agglomeration was microscopically (1000×) observed at the concentration used (0.04 mg/mL). The method of quantification of the carbon uptake is limited to particles with diameters greater than 0.5 μm. No significant differences in the kinetics of the carbon black uptake were observed between the two carbon black samples. The uptake was linear ($R^2 > 0.971$) with time, which suggests that carbon black uptake by the macrophages was pseudo-first order. This is a reasonable result since the number of carbon black particles is much greater than the number of alveolar macrophages.

Both adsorbates suppressed the phagocytosis of carbon black N339 at monolayer coverages of 0.5. In contrast, adsorbate coverages of 1.0 monolayers had no significant effect on the phagocytic ingestion of N339. The increased carbon black uptake of particles with 1.0 monolayer of adsorbates, as compared to carbon particles with 0.5 monolayer of adsorbates, may be an anomalous result due to the method of determining the carbon black particle uptake. Once the carbon black particles are covered with the adsorbate at a coverage of 1.0 monolayer, they are less likely to agglomerate to particles with a greater size than 30 nm and, therefore, they are difficult to visualize with an assay that is limited to those particles with diameters >0.5 μm. This hypothesis is supported by the data obtained with the reserve capacity of the alveolar macrophage to phagocytize a secondary particle, which show decreases in phagocytosis as the coverage of the adsorbate increases.

When carbon black BP 2000 was sorbed with either 0.5 or 1.0 monolayer of the adsorbates, the phagocytic ingestion of BP 2000 was not impaired. The heats of adsorption of the two adsorbates acrolein and benzofuran for the carbon black BP 2000 are greater than the heats of adsorption of the same adsorbates for the carbon black N339 (*12*). These results suggest that it will be more difficult to remove the adsorbates from the surface of BP 2000 to affect carbon black uptake. This result is very important since it should be emphasized that there is 15 times more adsorbate on the surface of BP 2000 than there is on N339 due to the greater specific surface area of BP 2000. Therefore, these results show that the coverage of the adsorbate on the surface of the carbon black is more important in defining the carbon black uptake than the absolute amount of adsorbate. At these low coverages, i.e., under the first equivalent monolayer, the adsorbates used in this study were not released by physical processes into the culture medium (*8*).

Having studied the role of the adsorbates on the phagocytic ingestion of carbon blacks, the next series of experiments was designed to elucidate the effect of the carbon black-adsorbate complexes on the reserve capacity of the alveolar macrophages to phagocytize a secondary particle. The data demonstrate that when the macrophages were pretreated with carbon black N339 with both adsorbates at both coverages, the capacity of the phagocyte to ingest a subsequent particle challenge was significantly impaired. In contrast, when macrophage pretreatment was with carbon black BP 2000-adsorbate complexes, the phagocytosis of the secondary particle was not impaired. The observation that secondary particle phagocytosis was not affected by the coated or uncoated BP 2000 samples suggests that the adsorbates are not bioavailable in 8 hr from this carbon black via metabolism.

The results of the longer term treatments (Fig. 5) indicate that carbon black-adsorbate complexes also prevent *in vivo* activation of macrophages as defined by enhanced Fc-receptor-mediated phagocytosis. During the course of these experiments, it was found that continued incubation of the alveolar macrophages in media alone sequentially increased Fc-receptor-mediated phagocytosis. For example, the phagocytic index remained at approximately 650 for the first 8 hr of incubation of the macrophages. Thereafter, prolonged incubation of the macrophages continually increased phagocytosis until at 72 hr the phagocytic index was approximately 1400. Treatment of the macrophages with carbon black N339 for 8 hr did not impair the increased phagocytic potential of the macrophage (Fig. 5). In contrast, coverage of N339 with 0.75 monolayer of acrolein interfered with the development of enhanced phagocytosis.

As previously stated, in the distal lung the alveolar macrophage phagocytic system serves as the primary defense mechanism against inhaled particles that reach the distal lung (*3*). In the alveoli, the macrophages engulf the deposited particles, thereby sequestering them from the vulnerable respiratory membrane. The data herein demonstrate that alveolar macrophage phagocytosis of particles can be impaired under at least some conditions by the presence of adsorbates on their surface. Secondly, the ingestion of the particle-adsorbate complex impairs the capacity of the macrophage to phagocytize a subsequent particle challenge. This impaired capacity of the macrophages to phagocytize the primary (carbon black-adsorbate complex) and the secondary particle may result in a breach of the defenses in the deep lung.

The outcome of this event could take several paths. For example, the increased residence time of particles on the alveolar surface may provide the opportunity for particles to penetrate the interstitium which could, in turn, result in a pathologic outcome. Alternatively, phagocytic dysfunction may lead to an increased susceptibility to pulmonary infections. Finally, the alveolar macrophages consist of a heterogeneous population (*13*) functioning not only as phagocytes but also as accessory cells with regulatory functions (*14*) that include the synthesis and release of numerous bioactive compounds (*15,16*) and the processing and presentation of antigen to lymphocytes for the establishment of the specific immune response (*17,18*). Whether or not these nondefensive functions of the macrophages are also affected by exposure to carbon black-adsorbate complexes remains to be elucidated.

The results of this study show that the surface properties of the particles, the chemical properties of chemical pollutants, and the interactions between particles and pollutants could play a major role in defining the biological effects of particles that are carried to the distal lung.

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