In Vivo Particle Uptake by Airway Macrophages in Healthy Volunteers

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We combined two techniques, radiolabeled aerosol inhalation delivery and induced sputum, to examine *in vivo* the time course of particle uptake by airway macrophages in 10 healthy volunteers. On three separate visits, induced sputum was obtained 40, 100, and 160 min after inhalation of radiolabeled sulfur colloid (SC) aerosol (Tc99 m-SC, 0.2 μ m colloid size delivered in 6- μ m droplets). On a fourth visit (control) with no SC inhalation, induced sputum was obtained and SC particles were incubated (37°C) *in vitro* with sputum cells for 40, 100, and 160 min (matching the times associated with *in vivo* sampling). Total and differential cell counts were recorded for each sputum sample. Compared with 40 min ($6 \pm 3\%$), uptake *in vivo* was significantly elevated at 100 ($31 \pm 5\%$) and 160 min ($27 \pm 4\%$); both were strongly associated with the number of airway macrophages ($R = 0.8$ and 0.7 , respectively); and the number and proportion of macrophages at 40 min were significantly ($P < 0.05$) elevated compared with control ($1,248 \pm 256$ versus 555 ± 114 cells/mg; $76 \pm 6\%$ versus $60 \pm 5\%$). Uptake *in vitro* increased in a linear fashion over time and was maximal at 160 min (40 min, $12 \pm 2\%$; 100 min, $16 \pm 4\%$; 160 min, $24 \pm 6\%$). These data suggest that airway surface macrophages in healthy subjects rapidly engulf insoluble particles. Further, macrophage recruitment and phagocytosis-modifying agents are factors *in vivo* that likely affect particle uptake and its time course.

Keywords: airway macrophages; induced sputum; mucociliary clearance; radiolabeled particles

Over the past several years, induced sputum has become a method of choice to noninvasively assess markers of airways inflammation in human subjects. We have previously demonstrated that when compared with bronchoalveolar lavage (BAL)-derived cells, sputum cells (macrophages, monocytes, and neutrophils) are equally viable and functional, with respect to phagocytic capacity, oxidative burst generation, and expression of cell-surface receptors associated with inflammation and innate host defense (1). We also showed, through the use of radiolabeled aerosol bolus delivery techniques, that induced sputum retrieves samples selectively from the surfaces of the bronchial airways as compared to peripheral airways (2). In healthy individuals, the predominant cell type recovered in sputum samples are macrophages, followed by neutrophils (3). Due to their surface location, these cells represent one of the first lines of cellular defense against inhaled pathogens from the external environment. For this reason, sputum cells are ideal for under-

standing how airway surface phagocytes interact with inhaled particles.

Traditionally, the bronchial airways in human volunteers have been a difficult region of the lung to examine in a noninvasive fashion. Hence, both quantitative and qualitative data to characterize the cellular and biochemical events that occur within them is limited. As a result, information on the functional properties of phagocytic cells (in particular their ability and time course for taking up particles), and the effect of macrophage numbers on these parameters, is lacking, especially when it comes to examining these properties in a dynamic, human *in vivo* model. Experimental techniques such as electron microscopy for *in situ* studies have been used mainly in animal models to examine phagocytic properties of lung cells (4–8). These studies have provided valuable information on the properties of lung phagocytes, although they require the use of special isolation and fixation techniques that prevent the examination of events in “real time.” A recent review (9) of the morphologic aspects of particle uptake by lung phagocytes in hamsters (10–12) and rats (13) show that engulfment of inhaled particles by airway macrophages is rapid, occurring as soon as 40 min after inhalation, and the process is essentially complete within 24 h. Macrophage recruitment to the site of particle deposition has also been noted to be rapid in these animal models, yet in hamsters, only a small proportion of the recruited cells (12–15%) are actively engaged in the phagocytosis of particles (7). These same *in vivo* analyses have not been conducted in humans after actual inhalation of particles. To better understand the etiologic processes of airway diseases caused by inhaled aerosols, it is important to first investigate the interaction between airway phagocytes and inhaled particles in healthy volunteers under normal homeostatic conditions.

The purpose of this study was to examine particle uptake by airway surface phagocytes, their time course of action, and the association between the number of macrophages and particle uptake in healthy volunteers. To accomplish this, we combined the use of induced sputum, a method that selectively retrieves macrophages and neutrophils from the surfaces of the bronchial airways in healthy individuals, with radiolabeled aerosol bolus delivery, a method that preferentially delivers, via controlled inhalation, traceable particles to the bronchial airways (2). In addition, we examined the difference between *in vivo* particle uptake after *in vivo* exposure (inhalation) versus *in vitro* particle uptake after *in vitro* exposure, as a means to understand the effect of the airway milieu on cell–particle interactions. Finally, we employed electron microscopy, fluorescence microscopy, and flow cytometry in ancillary studies to investigate the nature of the particle/cell association.

This study was approved by the Committee on the protection of the Rights of Human Subjects as the University of North Carolina (Chapel Hill, NC).

MATERIALS AND METHODS

Experimental Design

The experimental design is shown schematically in Figure 1. The study design comprised four distinct components: (1) inhalation of radiolabeled

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Figure 1. Schematic of the experimental design and study protocol. A timeline is shown indicating the three different time points that induced sputum was performed after cessation of radiolabeled aerosol inhalation.

particles (with deposition targeted to the central airways) and subsequent monitoring of mucociliary clearance (MCC) via γ scintigraphy; (2) assessment of *in vivo* uptake of inhaled radiolabeled particles by airway phagocytes recovered from induced sputum at specific time points after aerosol inhalation; (3) reproduction of the *in vivo* uptake protocol *in vitro* by co-incubating radiolabeled particles with sputum phagocytes for the same durations as the *in vivo* incubations; and (4) conducting of ancillary studies on selected sputum samples to establish the nature of the particle/cell association (i.e., particle internalization versus membrane adherence) using flow cytometry (FCM), epifluorescence microscopy (EFM), and electron microscopy (EM) techniques.

All 10 ($n = 10$) subjects were studied on three separate occasions, and 7 ($n = 7$) of the 10 were studied on a fourth separate occasion. All subjects were randomly assigned to their study occasions. On three occasions, subjects inhaled a radiolabeled (Tc99-SC) aerosol with subsequent monitoring of MCC and collection of induced sputum. Each of these sessions differed only as to when induced sputum was performed after cessation of radiolabeled aerosol inhalation. Induced sputum was performed either immediately, at 60, or 120 min after aerosol inhalation. Since the induced sputum procedure itself took 40 min to perform, 40 min is included in the induced sputum measurement times (min) referred to in this manuscript. Hence, the immediate measurement time is labeled 40 min, and the 60- and 120-min measurement times are 100 min and 160 min, respectively (Figure 1). During the fourth session, subjects ($n = 7$) did not inhale the radiolabeled aerosol, but underwent the induced sputum procedure to provide sputum cells for *in vitro* evaluation of particle uptake, as well as cells for FCM, EFM, and EM data.

Subjects

Ten healthy, nonsmoking volunteers aged 21 to 53 yr (5 male, 5 female) were recruited to participate in the study. All subjects received a medical exam on a separate screening day before beginning the study. Female subjects provided a urine sample for pregnancy testing. A positive pregnancy test resulted in exclusion from the study. All subjects had no history of chronic lung disease and had been free of upper or lower respiratory tract infections for 4–6 wk before beginning the study. All subjects had a forced expiratory volume in 1 s (FEV₁) of $> 80\%$ (91–118%) of predicted values for a population of similar height, weight, sex, age, and race. Informed written consent was obtained from all subjects before their participation in the study.

Radiolabeling Technique and Aerosol Generation

Radiolabeled (Tc99 m) sulfur colloid (SC, 5 mCi) was prepared by using Technescan Sulfur Colloid Kits (CIS-Sulfur Colloid; CIS-US, Inc., Bedford, MA) following the procedure provided by the manufacturer. The binding of Tc99 m to SC was always $> 99\%$ as determined by paper chromatography. The resultant Tc99 m-SC particles are sub-micronic, with a number mean diameter of 0.22 μm and geometric standard deviation (GSD) of 1.75 (14). Two milliliters of the particle suspension were placed in a modified Devilbiss 646 jet nebulizer (Devilbiss, Somerset, PA) and pulsed at 40 psi to generate 6- μm MMAD polydisperse (GSD = 2.40) aerosol droplets (i.e., aqueous particles containing the smaller suspended Tc99 m-SC particles). Because the nebulizer was located close to the subject's mouth, there was negligible evaporation and the aerosol was deposited on the airway surface as aqueous droplets and not as dry particles. After deposition, the Tc99

m-SC particles suspended within the droplets dissociated into 0.22- μm particles.

Inhalation of Radiolabeled Aerosol Boluses

A single-breath nitrogen washout test and a Xenon 133 equilibrium scan (rebreathing 1 mCi/liter) (Malinkrodt Medical, St. Louis, MO) were performed on the first visit before radiolabeled aerosol inhalation to determine anatomic dead space (ADS) and to determine the lung outline and volume of the lung, respectively (2).

Aerosols of radiolabeled SC particles were generated and delivered to the subject using a central airway deposition method that has been previously described in detail (2). Briefly, a small (40-ml) bolus of aerosolized particle suspension was delivered to shallow volumetric front depths (VFDs) in the lung of the subjects. By computer-controlled activation of the compressed air source used to nebulize the particle suspension, the boluses were delivered to a VFD of ~ 0.6 anatomic dead space (ADS) for each subject (mean $\pm$ SD VFD = 95 ± 22 ml, mean $\pm$ SD ADS = 154 ± 33 ml). The Fowler ADS was measured by single-breath nitrogen washout in the first six subjects, but due to equipment failure (i.e., the mass spectrometer) ADS was estimated in the last four subjects from a regression equation for ADS versus VC (vital capacity) derived from the first six subjects and those of our previous study (2) ($r = 0.88$). A photometer and pneumotachograph (Fleisch #1; Fleisch, Lausanne, Switzerland) was placed between the subject's mouthpiece and the nebulizer which measured the relative aerosol concentration and respired volumes for determining the VFD of each inhaled bolus. Despite potential errors in an individual's measured ADS, the VFD was fixed within an individual for each of the three study days. After inhalation of each bolus under controlled conditions (500 ml tidal volume at 125 ml/s flow) the subject held his/her breath for 5 s, followed by a rapid exhalation to maximally deposit the particles on the bronchial/conducting airway surface. In general, inhalation of 20–30 boluses over a 15-min period were required to deliver a sufficient activity to the lungs ($\sim 20 \mu\text{Ci}$), as monitored by a single crystal NaI scintillation detector placed at the subject's back.

Mucociliary Clearance Measurements

Mucociliary clearance (MCC) was measured by γ scintigraphy commencing immediately after SC aerosol inhalation on all three visits. Subjects rinsed their mouth with water after SC inhalation, then sat with their back to the γ camera (Elsint large-field-of view, SP-4 equipped with a high sensitivity collimator; Elscint, Haifa, Israel) for two initial 2-min scans and then remained seated in front of the camera for 2 h to monitor MCC. Monitoring of MCC was interrupted only for brief periods of time to perform the cough phase of the sputum induction procedure. MCC measurements resumed immediately after sputum inductions (i.e., at 40, 100, and 160 min; see Figure 1). All subjects returned to the laboratory the next day for a 30-min γ camera scan to assess retention of particles at 24 h after SC inhalation. The longer scan at the 24-h time point provided better counting statistics after the 3 to 4 half-life decay of Tc99 m.

Analysis of Deposition Patterns, Particle Clearance, and Normalization of Sputum Counts

To assess the degree of central (C) versus peripheral (P) airway deposition within the lung for all three study days in each subject, we calculated a C/P ratio of Tc99 m activity, normalized to the Xenon 133 equilibrium

scan, on the initial deposition scan after Tc99 m-SC aerosol inhalation (2). To eliminate activity associated with the stomach in the left P region, it was necessary to exclude the lower left lung base when creating the left lung region-of-interest. This should have had no effect on our intrasubject C/P comparisons, since the same regions were used for all study days and the C/P was normalized to associated lung volumes (Xe 133 equilibrium) in each case. A rectangular region bordering the right and left lung (defined by the Xe133 equilibrium scan) was used to determine, by computer analysis, the whole lung retention/clearance as a percent of the initial counts (background and decay-corrected) over the γ camera scanning period of 2 h and at 24 h. Again care was taken to assure that the activity in the stomach was excluded from the left lung region.

Sputum Induction

On three separate study visits, induced sputum was performed over a 40-min interval, beginning either immediately, at 60, or at 120 min after inhalation of the Tc99-SC aerosol (Figure 1). On a separate (fourth) visit, induced sputum was performed without prior inhalation of the radio-aerosol, to obtain sputum cells for *in vitro* studies, FCM, EFM, and EM measurements. The induced sputum procedure has been previously described in detail (2). Briefly, subjects underwent two 12-min periods of hypertonic saline (5%) inhalation. At the end of each 12-min inhalation period, subjects performed a three-step cleansing procedure before cough attempts and sample expectoration, to minimize squamous epithelial cell contamination of the expectorated sample. The cleansing procedure involved rinsing the mouth with water, scraping the back of the throat and expectorating the waste (but not coughing), and blowing the nose to reduce post-nasal drip contamination. After expectoration of sputum into a specimen cup, the subject's FEV₁ was monitored as a safety endpoint and compared with pre-sputum induction FEV₁ values. The entire procedure required 40 min to complete.

Sputum Processing

Immediately after sputum induction, radioactivity within the unprocessed total sputum sample was measured by placing the sample container a specified standard distance (30 cm) from the face of a single crystal NaI scintillation detector for a 60-s count of radioactivity. Samples were then processed as has been previously described (1, 2). After recording the weight of the entire sputum sample, a cell-enriched "select" sample was obtained using thumb forceps to pluck visible clumps of cells and cell-rich mucus "plugs" from the raw sample to separate them from the noncellular portions of the sample. The nonselected portion of the sputum sample was not processed because these secretions typically contain nonphagocytic epithelial cells (> 90% squamous epithelial cells) and are considered to not be lung-associated, but rather originate from the oral-pharyngeal region. The weight of the select sample was recorded and radioactivity in the select sample was then measured as described. To break down mucus and extract sputum cells from the mucoid sputum sample, a volume of 0.1% dithiothreitol (DTT, Sputolysin; Calbiochem, San Diego, CA) in Dulbecco's phosphate-buffered saline (DPBS), equal to four times the volume of the selected sample, was added to the sample and tumbled for 15 min. After 15 min, an additional volume of DPBS (4 $\times$ volume of select sample) was added, resulting in a final 1:9 dilution of the select sample. The diluted sample was then counted as above, centrifuged (500 $\times$ g, 10 min), and the supernatant carefully transferred to a separate container using a micropipette to remove as much as possible without disturbing the cell pellet. The radioactivity in the pellet and supernatant was then counted separately as above and the cell pellet was then resuspended in 1 or 2 ml of Hanks' balanced salt solution (HBSS) and filtered through 54- μ m mesh gauze to remove contaminating squamous cells. A 10- μ l aliquot was diluted 1:2 with the vital stain, Trypan Blue, and counted in a Neubauer hemacytometer to quantify cells and assess viability. The cells were then diluted to a final concentration of either 1 or 2 $\times$ 10⁶ cells/ml for *in vitro* assays (depending on the assay). Differential cell counts were performed by light microscopy of cytocentrifuged cell preparations on glass slides and counting 300–400 leukocytes at $\times$ 800 magnification. Differential cell counts derived from sputum samples obtained for the *in vitro* phagocytosis assays of radiolabeled SC served as control values.

Assessment of *In Vivo* Particle Phagocytosis

The *in vivo* phagocytosis of SC particles by airway phagocytes was estimated by quantifying the radioactivity (as described above) associated with the cell pellet and that remaining in the supernatant after centrifugation of the DTT-treated select sputum sample. The percentage of radiolabeled particles left in the supernatant (% supernatant) after centrifugation was determined as follows:

$$\% \text{ Supernatant} = \frac{(\text{Supernatant counts}) \times 100\%}{\text{Pellet} + \text{Supernatant counts}} \quad (\text{Eq. 1})$$

A correction factor was applied to all % supernatant values to account for a small proportion of free radiolabeled particles that tended to spin down with the cell pellet after low-level centrifugation (500 $\times$ g, 10 min). The correction factor was based on a series of separate *in vitro* experiments in which the labeled SC particles were added to sputum cell suspensions after processing (i.e., after reduction with DTT [see below]) and immediately centrifuged (500 $\times$ g, 10 min). The mean percent of particles remaining in the supernatant for several such experiments was 85 (i.e., 15% of particles were spun down with cells). This mean percentage was used to normalize the % supernatant (Eq. 1) to approximate the true percentage of particles that were actually cell-associated (i.e., % uptake) as follows:

$$\% \text{ Uptake} = 100 - \left(\frac{100 \times \% \text{ Supernatant}}{85} \right) \quad (\text{Eq. 2})$$

Although some radioactivity was detected in the nonselected sample portions (on average < 15%), this likely resulted from non-cell-associated free SC particles. Thus, their exclusion from uptake calculations on the selected sample may have resulted in a modest overestimation (maximum of 15%, as a relative amount) of uptake. Separate experiments determined that DTT had no effect on the integrity of the Tc99 m label to remain affixed to the SC particles.

Normalization of Sputum Radioactivity

The radioactivity measured in the select sputum samples (described above) by the scintillation detector was also dependent on the total amount of activity initially deposited and still remaining in the lung at the time of sputum induction. To determine if comparable sampling of particles occurred on the three study days within a given subject, it was necessary to normalize these sputum counts relative to the amount of activity in the lung just before induction of sputum. Calculation of normalized sputum counts (NspC) was performed as follows:

$$\text{NspC} = \frac{\text{measured select sputum counts whole}}{\text{lung counts immediately prior to IS}} \quad (\text{Eq. 3})$$

The whole lung γ camera counts were determined from the rectangular regions bordering the left and right lungs (described above).

In Vitro Assessment of Radiolabeled SC Particle Uptake by Sputum-Derived Phagocytes

For comparison to *in vivo* particle uptake by airway phagocytes, we performed particle uptake experiments *in vitro*, using sputum-derived cells from 7 of 10 of the same subjects, which had provided *in vivo*-derived data. For these experiments, the subjects did not inhale radiolabeled aerosol. Immediately after sample processing, 5 $\pm$ 2 drops (5–20 μ Ci) of Tc99 m-SC particle suspension was added *in vitro* to a suspension of sputum cells (1 $\times$ 10⁶ cells/ml in HBSS). The cell/particle suspension was then gently mixed and subsequently incubated (37°C) with periodic mixing. To simulate the time frames for collection of sputum for *in vivo* uptake measurements, equal aliquots (one third of total volume) of the suspension were removed at 40, 100, and 160 min (corresponding to the *in vivo* "incubation" intervals for the same individual), each time after gently mixing the incubating suspension. These were centrifuged (500 $\times$ g, 10 min) to obtain the cell pellet and supernatant fractions, the latter carefully pipetted into a naïve test tube. Counts of radioactivity were measured on both the cell pellet and supernatant fractions as described and the % uptake calculated (Eq. 2)

Fluorescence Microscopy and Flow Cytometric Evaluation of *In Vitro* Particle Uptake

While our uptake measures using Tc99 m-SC showed the particles to be associated with the cell pellet, we could not differentiate whether or not they had been internalized or were merely attached to the cell surface. To investigate the nature of the particle/cell association, we employed nonradioactive, fluorochrome-labeled SC particles, FCM, and EFM to perform separate *in vitro* experiments using sputum cells from two subjects. A suspension of SC was prepared using a kit (CIS-Sulfur Colloid; CIS-US) in accordance with the manufacturer's instructions, except that 1 ml normal saline solution (nonradioactive) was substituted for the radioactive technetium (Tc^{99m}) label. The suspended sulfur colloid particles were labeled with fluorescein isothiocyanate (FITC) using a Fluoreporter FITC Labeling Kit (Molecular Probes, Eugene, OR) in accordance with the manufacturer's instructions. A 200- μ l aliquot of the sulfur colloid solution was labeled using a dye to protein molecular ratio of 50, assuming a $MW_{protein}$ of 330 kD for gelatin. One hundred microliters of FITC-labeled SC particle suspension was incubated in a polypropylene tube with 100 μ l of a suspension (2.0×10^6 cells/ml) of sputum-derived leukocytes in HBSS at 37°C for 1 h. After incubation, cells were fixed by adding 200 μ l of 2% formaldehyde (EM grade; Electron Microscopy Sciences, Ft. Washington, PA) to the mixture. EFM was performed using appropriate filters on a Zeiss Axiovert-10 fluorescence microscope (Zeiss, Thornwood, NY), using unstained and uncoverslipped cytocentrifuged cell preparations. Control slides were prepared using cell suspensions incubated without SC.

Flow cytometry was performed on these same cells to further evaluate phagocytosis of SC particles. The procedure for FCM examination of sputum leukocytes has been previously described in detail (1). Briefly, flow cytometry was performed with a FACSORT (Becton Dickinson, Franklin Lakes, NJ) using an Argon-ion laser (wavelength = 488 nm). Gain and amplitude settings were set to accommodate sputum samples, which allowed for the establishment of reference gates for leukocyte identification. Gating of viable macrophages, monocytes, neutrophils, and lymphocytes in sputum was based on light scatter (FSC/forward scatter, SSC/side scatter) properties and positive cell surface expression of CD45 (pan-leukocyte marker), with further confirmation by the expression of CD16 (neutrophils), HLA-DR/CD14 (mononuclear phagocytes), and CD3 (lymphocytes). All fluorescent antibodies were obtained from a single commercial source (Beckman Coulter, Inc., Miami, FL: CD45 cat# IM20782, CD14 cat# IM0650, CD16 cat# IM1238 and CD3 cat# IM1282). Phagocytosis of FITC-SC was assessed by measuring changes (histogram analysis) in mean fluorescence intensity (MFI) of SC-FITC-exposed sputum phagocytes and a shift in light scatter properties of phagocyte populations as compared with controls.

Electron Microscopy and Electron Energy-Loss Spectroscopy

To further establish the intracellular location of SC particles, selected samples of sputum leukocytes obtained after inhalation of Tc99 m-SC were examined by energy filter transmission electron microscopy (EFTEM) and electron energy loss spectroscopy (EELS). Chemically fixed cells (2% formaldehyde, EM grade) were embedded in epon, cut into ultrathin sections of < 50 nm, transferred onto uncoated 600-mesh copper grids, and post-stained with uranyl acetate and lead citrate. Ultrathin sections were examined in a LEO 912 transmission electron microscope equipped with an in-column energy filter for elemental microanalysis. For elemental identification of sulfur, the $L_{2,3}$ -edge at 164.8 eV energy loss was used.

Cell Morphology

Morphology of sputum macrophages was assessed by light microscopy, and macrophages were classified as having either normal to minimal degenerative morphology or moderate to advanced degenerative morphology. Macrophages were considered to have moderate to advanced degenerative morphology if they exhibited two or more of the following morphologic changes: (1) cytoplasmic swelling and vacuolar (hydropic) degeneration; (2) alterations of the plasma membrane (blebbing, rupture); (3) degenerative nuclear changes (nuclear swelling, karyolysis or karyorrhexis, loss of nucleoli, disruption of nuclear membrane); (4) altered (diminished) cytoplasmic and nuclear staining characteristics. Normal and degenerative macrophage populations were expressed as

a percentage of total macrophages (based on 200 cells counted per slide).

Statistical Methods

Statistically significant differences between multiple study end-points were assessed using a repeated-measures one-way ANOVA. Paired mean analysis between two study end-points was assessed using paired parametric (*t* tests) or nonparametric (Wilcoxon matched pairs) tests as appropriate. Simple linear regression analysis and Pearson correlation coefficients (*R*) were used to assess associations between endpoints. For all statistical tests, a *P* value of < 0.05 was considered statistically significant.

RESULTS

Regional Particle Deposition, MCC, and Normalized Sputum Counts

The mean ($\pm$ SD) C/P ratios for the three study days (i.e., 40, 100, and 160 min) were 1.91 ± 0.50 , 1.81 ± 0.43 , and 1.77 ± 0.36 , respectively, and were not different when analyzed by repeated measures tests. This suggested that the particles were similarly deposited in the bronchial airways for the three study periods. The average retention versus time curves on the three study days for all subjects is illustrated in Figure 2. About 40–50% of the deposited activity was cleared from the lung by induced sputum at the 40-min time point. Because lung MCC occurred naturally prior to the induced sputum procedures initiated at 100 and 160 min after aerosol inhalation, the actual amount cleared via induced sputum at the 60–100 and 120–160 min time intervals was reduced accordingly. Interestingly, at the conclusion of the induced sputum for each time point, the lung retentions were similar (0.48–0.52) and approximated those observed 24 h after deposition (0.40–0.42) of the radiolabeled particles. This suggests that at each time point, sputum induction cleared the airways of nearly all “clearable” particles. There were also no correlations between lung retentions immediately before sputum induction (for the 100- and 160-min samples) and the measured %uptake of particles by airway phagocytes that might suggest preferential clearance of free versus phagocytosed particles before sputum induction. Furthermore, normalized sputum counts (NSpC) were not different between the three time points for induced sputum (NSpC = 0.67 ± 0.41 , 0.60 ± 0.66 , and 0.65 ± 0.66), suggesting comparable sampling of the airways relative to the available particle activity in the lung at the time of sputum induction.

Figure 2. Average retention versus time curves for all subjects and the three study days. Forty to fifty percent of the deposited activity was cleared from the lung by induced sputum at the 40-min time point.

Sputum Characteristics, Total and Differential Cell Counts

All subjects tolerated the induced sputum procedure without experiencing any adverse events or need for rescue therapy. All ten subjects were able to produce a sputum sample of adequate quality ($\geq 60\%$ viability, $\leq 20\%$ squamous epithelial cells) on all study occasions. The mean number and proportion (%) of airway macrophages (and neutrophils) obtained at each time point (40, 100, 160 min) after sulfur colloid (SC) inhalation and after the control condition, appear in Table 1. After particle inhalation, the mean ($\pm$ SEM) absolute number of macrophages was maximal at 100 min ($1,523 \pm 246$ cells/mg), but was not significantly different than levels at 40 ($1,248 \pm 256$ cells/mg) or 160 min (990 ± 211 cells/mg). The proportion (%) of macrophages, however, was significantly ($P < 0.05$) elevated at 100 ($92 \pm 2\%$) and 160 ($92 \pm 2\%$) min compared with 40 min ($76 \pm 6\%$). Compared with control (555 ± 114 cells/mg and $60\% \pm 5\%$), both the number and proportion of macrophages at 40 min were significantly ($P < 0.05$) elevated ($1,248 \pm 256$ cells/mg and $76\% \pm 6\%$), suggesting that SC inhalation induced macrophage recruitment or accumulation in the airways. Macrophage numbers peaked at 100 min ($1,523 \pm 246$ cells/mg) and decreased at 160 min (990 ± 211 cells/mg) versus control, suggesting that macrophage accumulation was likely complete shortly after 100 min post-aerosol inhalation.

In Vivo and In Vitro Uptake of Radiolabeled SC Particles

Mean ($\pm$ SEM) *in vivo* particle uptake (%) at 40, 100, and 160 min after SC inhalation is shown in Figure 3A. Uptake was significantly ($P < 0.05$) enhanced at 100 ($31 \pm 5\%$) and 160 min ($27 \pm 4\%$) compared with 40 min ($6 \pm 3\%$), and was maximal at 100 min. Mean ($\pm$ SEM) *in vitro* particle uptake after *in vitro* exposure is shown in Figure 3B. *In vitro* uptake was maximal and significantly elevated at 160 min compared with the 40-min time point ($24 \pm 6\%$ versus $12 \pm 2\%$, $P < 0.05$). Uptake *in vitro* increased linearly over time from 40–160 min after the application of SC particles, and compared with uptake *in vivo* was significantly elevated at the early time point of 40 min ($12 \pm 2\%$ versus $6 \pm 3\%$, $P < 0.05$).

EFM and FCM

Light microscopic and EFM photomicrographs showing the uptake of SC-FITC particles by sputum macrophages after *in vitro* incubation are depicted in Figure 4. *Top* (light microscope) and *bottom* (fluorescent microscope) *right panel* images (*C* and *D*) show particles as bright/fluorescent areas in contrast to the dark background of the cell. These images are compared with their respective *left-hand panel* images, in which cells were not exposed to particles (control condition) and the absence of bright/fluorescent areas is noted. The fluorescence is confined to the cytoplasm of macrophages and neutrophils and is absent in squamous cells and lymphocytes (not shown). This shows a definite association of the particles with phagocytic cells. The diffuse

distribution within the cytoplasm (as opposed to localization at the cell periphery) suggests an intracellular location for the particles, rather than extracellular surface attachment.

Flow cytometric analysis of phagocytosis of FITC-labeled SC particles by sputum macrophages is shown in Figure 5. A representative *dot plot* (*A*) in the control condition (no particle inhalation) shows the gated sputum leukocyte populations (macrophages, neutrophils, monocytes, lymphocytes) based on light scatter properties (*x*-axis: forward scatter; *y*-axis: side scatter). A corresponding *histogram* (*B*) shows the different cell population sizes (forward scatter) relative to the small size of the SC particles, which fall below the threshold limit of 200 MFI for the cells. Phagocytosis is demonstrated in *C*, where macrophages have ingested SC particles (*green peak*) and have shifted to the right into the particle region (M3) away from the control region (M1-*pink peak*), where only background autofluorescence is present and no particle uptake occurred.

Electron Microscopic Studies

On EM analysis, particles were found mostly as agglomerates surrounded by a halo that contained no or only little sulfur. The particles themselves revealed the specific sulfur signal using electron energy loss spectroscopy (EELS). Particles were localized within macrophages and the majority had a diameter of 100 nm or more, suggesting agglomeration of the primary particles within the cells. Figure 6 is a transmission electron micrograph depicting an airway macrophage containing an intracellular SC particle after particle inhalation *in vivo*. At low magnification (*A*), SC particles (*black aggregate mass* in *boxed region*) are located in the perinuclear region within the cytoplasm of a sputum macrophage. The *boxed region* in *A* is seen in higher magnification in *B*. The particle consists of a large irregularly shaped electron-dense mass that is an aggregate of smaller particles. The large particle mass is surrounded by an amorphous electron-lucent material (interpreted to be the gelatin matrix of the nebulized SC particle) and multiple smaller electron-dense particles organized around the periphery. It is not possible to discern whether the main particle mass is encased in a membrane-bound structure (phago-lysosome) due to inadequate staining and contrast of cellular membranes. Electron spectroscopic images (ESI) displayed the specific signal of sulfur in white pixels. The electron-dense particle aggregate, as well as the multiple smaller electron-dense particles organized around the periphery, depicted in Figure 6B, co-localize with the distribution of sulfur as detected using EELS shown by white pixels in Figure 6C. The sulfur-poor electron-lucent region surrounding the particle aggregate shows only a weak signal for sulfur.

Association between In Vivo Particle Uptake and Macrophage Levels in the Airways

Positive associations were observed between particle uptake (%) and the absolute number of macrophages (cells/mg) at both

TABLE 1. IN VIVO MEAN ($\pm$ SEM) SPUTUM MACROPHAGE AND NEUTROPHIL CELL COUNT AFTER Tc99 m SULFUR COLLOID PARTICLE INHALATION BY HEALTHY SUBJECTS

Time after Particle Inhalation	Macrophages/mg Sputum	% Macrophages	PMN/mg Sputum	% PMNs
Control (<i>n</i> = 7) (no particle inhalation)	555 (114)	60 (5)	325 (85)	35 (7)
40 min	1248* (256)	76* (6)	486 (356)	20 (9)
100 min	1523* (246)	92*† (2)	118 (28)	8 (2)
160 min	990 (211)	92*† (2)	82 (20)	8 (2)

Definition of abbreviation: PMN, polymorphonuclear leukocytes.

* $P < 0.05$ versus control.

† $P < 0.05$ versus 40 min.

Figure 3. (A) *In vivo* particle uptake (%) by airway macrophages obtained from induced sputum at 40, 100, and 160 min after radiolabeled (Tc99 m) sulfur colloid (SC) aerosol inhalation by healthy subjects ($n = 10$). Uptake was significantly elevated at 100 and 160 min compared with 40 min ($*P < 0.05$). (B) *In vitro* particle uptake (%) by airway macrophages obtained from induced sputum from $n = 7$ healthy subjects after incubation with radiolabeled (Tc99 m) sulfur colloid (SC) particles for 40, 100, and 160 min. Uptake was significantly elevated at 160 min compared with 40 min ($*P < 0.05$).

100 min ($R = 0.84$, $P = 0.01$) and 160 min ($R = 0.68$, $P = 0.04$). These associations and their respective regression lines are shown in Figures 7A and 7B. No association between uptake (%) and macrophage number was observed at 40 min after SC inhalation ($R = 0.04$). Positive associations were observed between particle uptake (%) and macrophage proportion (%) at 40 ($R = 0.4$), 100 ($R = 0.4$), and 160 ($R = 0.2$) min, but these did not reach statistical significance in each case. In addition, no associations were observed between particle uptake (%) and proportion or number of neutrophils at any time point. No significant association between *in vivo* and *in vitro* uptake was found at 100 and 160 min, but a trend toward an association was observed at 40 min ($R = 0.6$, $P = 0.06$, one-tailed).

Morphology

Our analysis on six subjects showed that on average ($\pm$ SEM) there were a higher proportion of macrophages demonstrating degenerative morphologic changes consistent with necrosis at 40 min ($67 \pm 2\%$) compared with 100 min ($39 \pm 4\%$). These changes were marked by of hydropic degeneration (extensive cytoplasmic vacuolation), cell swelling, and disruption of membranes resulting in blebbing and rupture of the plasma membrane, nuclear swelling, karyorrhexis, and karyolysis (fragmentation and dissolution of the nucleus). Cells at 40 min likely contained a smaller proportion of newly recruited cells, since

macrophage proportions were maximal at 100 min. Therefore, at 40 min there were likely a relatively higher proportion of older cells that would tend to have increased degenerative morphology. Cell morphology assessed before particle exposure (*in vitro* samples) showed a very similar proportion of degenerative cells ($64 \pm 3\%$) compared with cells at 40 min *in vivo*, suggesting that particles themselves were not inducing morphologic changes.

DISCUSSION

Combining the techniques of induced sputum with radiolabeled aerosol bolus delivery allowed us to dynamically assess particle uptake by airway surface macrophages in healthy volunteers. Unlike previous animal studies that used *in vitro* culture systems, or *in situ* methods on excised human lungs (15), the experimental approach used in this study examined cellular airways events after targeted aerosol inhalation to the central airways. This targeted approach from both a deposition and sampling perspective allowed us to specifically examine the bronchial airways independent of particle-alveolar macrophage interactions. Our controlled particle inhalation procedure also provided reproducible lung deposition on the three study days, guaranteeing comparable samples from induced sputum for evaluation of the time course of *in vivo* particle uptake by airway macrophages.

Figure 4. Uptake of fluorescent SC particles by airway macrophages obtained from induced sputum from a healthy subject. Images in the *top panels* (A, C) are shown under normal light microscopy, and those in the *bottom panels* (B, D) with fluorescent microscopy. *Top and bottom right panel* images show particles as bright/fluorescent areas in contrast to the dark background of the cell. Particle uptake by MØ (macrophages) and PMN (neutrophils) is shown in C and D. No particle uptake is seen in control cells in panels A and B. SQ (squamous epithelial cell).

Figure 5. *In vitro* phagocytosis of SC particles by airway macrophages obtained from induced sputum in a healthy subject. (A) Flow cytometric dot plot shows distribution of sputum leukocytes (PMN/polymorphonuclear neutrophils; MØ/macrophages; Mono/monocytes; LO/ lymphocytes) based on light scatter properties (FWS: forward scatter; SSC: side scatter). (B) Flow histogram shows SC particles register below FSC threshold of 200 MFI, while leukocytes register above 200 MFI. (C) Flow histogram shows rightward shift of macrophage population (green) into the M3 region after ingestion of SC particles. Control cells (no particle ingestion) are shown in pink in the M1 region and demonstrate only background autofluorescence.

Furthermore, the recovered sputum phagocytes did not require fixation for their analysis, but were rather processed in real time within their own functional milieu.

Comparing the time course kinetics between our *in vivo* and *in vitro* conditions, we observed some interesting differences in particle uptake. *In vivo*, uptake began but was low at 40 min, and was not maximal until 100 min after aerosol inhalation. By 160 min, uptake appeared to fall off slightly, but not significantly so. These time course kinetics are similar to animal time course studies in which uptake by hamster airway macrophages occurred as early as 40 min, and was usually maximal by 1 h after the onset of inhalation (7). *In vitro*, uptake was also evident at 40 min, but unlike *in vivo*, continuously increased over time, maximizing at 160 min. Together, these data suggest that in healthy human airways, there exists a population of resident macrophages on the airways surfaces that rapidly interact with inhaled particles. The nonlinear time course kinetics observed *in vivo* may reflect the presence of competing endogenous factors that can function to either enhance or inhibit particle uptake. For example, the surfactant film that covers the aqueous phase at the air-liquid interface may promote displacement of particles from air into an aqueous subphase (16–18). Here, specific factors that enhance uptake may be present and include surfactant proteins (SP) A and D and immunoglobulins, while others like 1,2-dipalmitoylphosphatidylcholine (DPPC) have adsorption-reducing effects on opsonic proteins and may inhibit particle uptake (19). These competing factors would be expected to be in low concentration in our *in vitro* model due to the removal of the supernatant fluid before particle incubation. Hence these cells received limited exposure to endogenous compounds from the airway's natural milieu, as well as no exposure to the potential effect of hypertonic saline on the airway milieu from the induced sputum procedure itself (20). The effect of endogenous factors on uptake, especially ones that enhance it, would be expected to be least at 40 min and greater at 100 and 160 min as they

require the necessary time to exert their actions. Hence, *in vivo* and *in vitro* uptake should be most similar early rather than later, when exogenous factor's influences are most minimal. Indeed, we observed a positive trend for association between *in vivo* and *in vitro* uptake at 40 min but not at 100 and 160 min. *In vivo*, competing factors would be secreted from endogenous stores within the airways and either directly or indirectly affect cell-particle interactions on the airways surface. The generally

Figure 6. Electron micrographic images of an ultrastructural view of intracellular SC uptake by airway macrophages obtained from induced sputum after *in vivo* particle inhalation by a healthy subject. (A) Sputum MØ containing intra-cellular SC Particle. (B) Detail showing substructure of the SC particle. (C) Electron Spectroscopic image showing specific sulfur signal.

Figure 7. Association between *in vivo* particle uptake and the number of sputum macrophages (macrophages/mg sputum) in healthy subjects 100 (A) and 160 (B) min after radiolabeled SC aerosol inhalation. Significant associations were observed at 100 (A; $R = 0.84$, $P < 0.05$) and 160 (B; $R = 0.68$, $P < 0.05$) min.

linear time course pattern we observed *in vitro* is more consistent with uniform reaction kinetics that has limited access to exogenous factors.

Maximal uptake *in vivo* was 31% for the observation times studied here. This is in the range of reported uptake values for hamster airway macrophages, where uptake ranged between 17 and 44% depending on the size and type of particle examined (9). Geiser (9) showed that the smallest particle type (3- μm spores) had the lowest uptake (10%), whereas the largest particle type (6- μm polystyrene microspheres) had the greatest uptake (44%). There is no comparative *in vivo* animal data for smaller colloidal particles (average 0.22 μm) such as those used in this study. While the effect of particle size on uptake in our human model would be of interest, there are limitations on the types and associated sizes of radiolabeled particles that human subjects can inhale. Moreover, the size of particles with which macrophages interact, will almost certainly fall into a range of sizes, since macrophages ultimately interact with both individual small-sized particles (0.22 μm), and larger agglomerates of the primary particles. We used flow cytometry and electron microscopy to show that sputum macrophages are indeed capable of phagocytosing SC particles, hence we are confident that our uptake measurements were a part of the phagocytosis process, not merely particle adhesion to cells.

After aerosol inhalation by healthy volunteers, the most significant increase in uptake occurred between 40 and 100 min after aerosol inhalation. There are several factors that may account for uptake differences *in vivo* versus *in vitro*, and one of these is macrophage recruitment. Macrophage recruitment to the site of

particle deposition likely influenced both absolute uptake and the time course of uptake at the early time point. *In vitro*, cell migration did not occur, since cell-particle interaction occurred immediately upon cell-particle incubation, whereas a significant portion of the early events *in vivo* likely involved macrophage recruitment to and organization at the site of particle deposition. During cell recruitment, optimal particle uptake would not have been expected to occur until both a critical number and proportion of macrophages were present and available for particle-cell interactions. Macrophage migration to sites of particle deposition after particle inhalation has been demonstrated *in situ* for several particle types and animal species (7, 21–23). Our differential cell count data suggest that macrophage recruitment did occur in subjects after particle inhalation, despite delivery of a very small mass of particles (< 10 μg) to the airway surface. There was a significant increase in both the number and proportion of macrophages after particle inhalation at 40 min when compared with a control condition (no particle inhalation). Furthermore, *in vivo* we observed that a significant increase occurred in the proportion of macrophages at both 100 and 160 min compared with 40 min, paralleling the significant particle uptake responses. Interestingly, particle uptake correlated positively and significantly with the number of macrophages at 100 and 160 min, but not at the 40-min time point. We interpret the absence of the correlation at 40 min, and the relatively low mean *in vivo* uptake value at 40 min (compared with *in vitro*), to be due in part to macrophage recruitment to the sites of particle deposition. This process and the time needed to achieve it would influence and likely delay full macrophage involvement in particle uptake at the early time point.

Morphologic analysis of the *in vivo* macrophage populations at both 40 and 100 min revealed that at 40 min, there was a higher proportion of macrophages that showed evidence of moderate to advanced degenerative cytoplasmic and nuclear morphology compared with the 100-min time point. This finding suggests that at the 40-min time point, a greater proportion of the airway macrophages are cells with longer residence times in the airways. These cells have reached or are approaching senescence, and have reduced functional ability compared with newly arrived monocytic cells from the peripheral circulation or interstitial macrophages from the peribronchial spaces (24). Taken together, our results suggest that by 100 min, when macrophage proportions *in vivo* were maximal (92%), cell recruitment and migration of new monocytic cells into the airway were complete. Consequently, macrophages with greater phagocytic ability were the predominant phenotype in the airway, enabling particle uptake processes to be optimal.

While most fine and coarse insoluble particles depositing on the bronchial tree are cleared by the mucociliary clearance apparatus (25), the role airway macrophage phagocytosis plays in particle clearance is less well understood. The fact that we found no association between clearance kinetics pre-sputum and the %uptake measured post sputum suggests that both free particles and particles within macrophages clear at similar rates. For example, if phagocytosis of particles inhibited clearance, as recently suggested by Moller and colleagues (26), we might have expected higher lung retentions pre-sputum for those individuals who exhibited higher %uptake after sputum. On the contrary, if free particles cleared less well than those taken up by macrophages (e.g., due to interaction or uptake by epithelial cells), we might have expected higher lung retentions pre-sputum for those individuals who exhibited lower *in vivo* uptake. The fact that no correlations occurred at all suggests that free and phagocytosed particles clear similarly within the mucociliary clearance apparatus. In addition, the fact that subjects achieved similar lung retentions post sputum at each collection time point (Figure 2)

suggests that “incubation” of particles on the airway surface for periods up to 2 h does not affect their ability to be cleared. This contradicts the hypothesis of Moller and colleagues (26), who suggested that uptake of particles by airway macrophage may be responsible for retarded clearance from the bronchial airways. Our data suggested that particle uptake by airway macrophages had no effect on clearance kinetics of particles through 24 h after deposition.

One limitation of this study was our inability to determine whether the retention of particles immediately post sputum or at 24 h after aerosol inhalation represented a predominance of free versus phagocytosed particles on alveolar regions. Previous studies (27) using similar particle inhalation techniques suggest that some of the particles still retained after sputum induction and at 24 h may reflect particles residing in small bronchiole airways where the induced sputum procedure may not sample (2), and whose clearance occurs over a period of weeks. We speculate that most of the particles retained at 24 h in this study are indeed likely residing in alveolar regions reached by the bolus delivery method (i.e., short path length alveoli), despite our best efforts to maximize bronchial airway deposition. These particles would be unavailable for sampling by the induced sputum technique (2). Moreover, we know from our previous studies that *in vivo* uptake of particles by alveolar macrophages also occurs very rapidly, with 90% uptake occurring by 24 h after instillation (28).

The results reported here describe the time course and characteristics of phagocyte–particle interactions on the surfaces of the bronchial airways in normal healthy individuals. These results will serve as useful comparisons to determine whether these host defense features are altered in airway diseases like asthma or COPD, in which inflammation plays a significant role in the pathophysiology of the disease. Furthermore, understanding particle uptake by airway macrophages may contribute toward therapeutic strategies, some of which try to enhance uptake to defend against toxic particles that may damage host tissue, while others try to inhibit particle uptake to enhance drug bioavailability to target cells and organs. Our technique for evaluating *in vivo* uptake of particles in the airways should provide a valuable tool for future studies to address these important questions.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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