Induced Sputum Derives from the Central Airways
Confirmation Using a Radiolabeled Aerosol Bolus Delivery Technique

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Indirect evidence suggests that induced sputum derives from the surfaces of the bronchial airways. To confirm this experimentally, we employed a radiolabeled aerosol bolus delivery technique that preferentially deposits aerosol in the central airways in humans. We hypothesized that there would be significantly more radioactivity recovered in an induced sputum sample, and greater airways clearance of radiolabeled particles, immediately after a central versus peripheral airways deposition. Ten healthy volunteers underwent radiolabeled aerosol deposition (^{99m}Tc sulfur colloid particles) to the central and peripheral airways on separate occasions followed immediately by induced sputum or no sputum (control), while seated in front of a gamma camera. Radioactivity was measured in the selected sputum sample, processed cell pellet, and supernatant fraction. Significantly more radioactivity was present in all portions of the sputum sample after central versus peripheral airways deposition (i.e., selected sample: 15,607 counts ± 2,985 versus 943 counts ± 298, p = 0.001). Clearance from the whole lung was significantly greater 40 min after central versus peripheral airways deposition (48 ± 3% versus 5 ± 1%, p = 0.0001). Compared with control, induced sputum greatly enhanced clearance after central deposition (48 ± 3% versus 11 ± 6%, p = 0.0001), but not after peripheral deposition (5 ± 1% versus 3 ± 0.8%). These results provide direct evidence that induced sputum derives from the central airways with little or no contribution from the peripheral airways.

Keywords: induced sputum; radiolabeled aerosol bolus

Induced sputum by inhalation of hypertonic saline has been used as a less invasive technique to examine airways inflammation in healthy (1), allergic (2), and asthmatic individuals (3, 4). Indirect evidence supports the assumption that induced sputum selectively samples the surfaces of the bronchial airways, while obtaining little or no secretions from the peripheral airways. Studies that have compared induced sputum samples with bronchoalveolar lavage (BAL) in healthy subjects at rest demonstrate that sputum samples contain a significantly greater percentage of neutrophils, an observation considered to reflect airways phenomena, whereas samples collected from airways regions such as proximal bronchial secretions, tracheal secretions, and bronchial wash (BW) have similar leukocyte profiles to induced sputum (1, 5–8). We illustrated these observations pictorially using quantitative flow cytometry in a recent study (1). In that study, we also showed that macrophages and neutrophils recovered from induced sputum have modified surface phenotypes and functional capacity compared with the same cells from BAL (1), suggesting that these cells derive from different lung compartments.

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Moreover, several studies have shown that induced sputum contains a higher concentration of fluid-phase components, such as eosinophil cationic protein (ECP), mucinlike glycoprotein, and albumin compared with BAL (6–9). These soluble components are thought to be present largely in airway secretions compared with alveolar spaces. Finally, cough is a critical component in the sputum induction process. Clinical studies examining airway clearance and deposition show that mucus clearance by cough is highly effective predominantly in the most proximal conducting (central) airways (10). All these studies indirectly suggest that induced sputum selectively samples the central airways, but there is no direct evidence to support this. Thus, the goal of this study was to directly test this hypothesis using quantifiable end points.

To achieve this aim, we used a novel aerosol generation and delivery system to examine airway deposition and clearance in human volunteers (11–14). This system has recently been used to better characterize particle clearance kinetics from the conducting airways by delivering small-volume (40-ml) aerosol boluses to shallow volumetric lung depths (i.e., less than 150 ml). We have further refined this technique to deliver jet-nebulized boluses of radiolabeled (^{99m}Tc) sulfur colloid (SC) particles within large droplets in order to target their deposition in the conducting airways, hereafter referred to as central airways. Clearance kinetics of these centrally deposited particles can also be compared with clearance from distal alveolar airspaces, hereafter referred to as peripheral airways, after large tidal volume inhalation of fine droplets containing the radiolabeled sulfur colloid particles. Using these novel aerosol delivery techniques, we hypothesized that if induced sputum derives primarily from the central airways, this will be reflected as significantly greater radioactivity recovered in the sputum sample immediately after a central versus peripheral airway deposition. Furthermore, if induced sputum is indeed an airways phenomenon, clearance of radiolabeled particles from the central airways should be markedly demonstrated immediately after a central airways deposition. Likewise, little or no clearance should be observed immediately after a peripheral airways deposition.

METHODS

Subjects

Ten healthy, nonsmoking volunteers 18 to 40 yr of age (4 male, 6 female) were recruited to participate in the study. All subjects received a medical examination on a separate screening day before beginning the study, and informed written consent was obtained. 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 lung disease (asthma, fibrosis, chronic obstructive pulmonary disease) and had been free of upper or lower respiratory tract infections for 4 to 6 wk before beginning the study. All subjects had a forced expiratory volume in one second (FEV_1) of greater than 80% of predicted values for a population of similar height, weight, sex, age, and race. Ethics approval for this study was obtained from the Committee on the Protection of the Rights of Human Subjects, School of Medicine, University of North Carolina–Chapel Hill.

Study Protocol

Each subject received four radiolabeled aerosol depositions (two peripheral and two central) on four separate occasions. Each radiolabeled particle exposure, whether central or peripheral, was approximated to be less than 20 μCi of the radioisotope technetium-99m (bound to SC) deposited in the lungs. Sputum induction was performed immediately after one central and one peripheral deposition. On each occasion, mucociliary clearance was measured over a 2-h period and at 24-h by gamma camera imaging (Elscint large-field-of-view, SP-4; Israel). A single-breath nitrogen washout test and a xenon-133 equilibrium scan (rebreathing 1 mCi/L) were performed on the first visit before an aerosol deposition to determine anatomic dead space (ADS) and lung volume imaging to normalize regional deposition, respectively (12). Single-breath nitrogen washout was measured from 500 ml above FRC according to the technique of Fowler (15). Subjects inhaled a single breath of 100% O_2 from residual volume to FRC + 500 ml and exhaled again to residual volume (RV). A special Teflon mouthpiece designed to reduce the volume of the oral cavity was used for each individual. This same mouthpiece was used for the radiolabeled bolus inhalations described subsequently. The exhaled volumes associated with the ADS (exhaled volume through the midpoint of phase 2 of the nitrogen washout curve, as determined by the equal area technique [15]) were the average of three nitrogen washout curves for each subject. Next, subjects received a randomly assigned peripheral or central (bolus) aerosol deposition with or without sputum induction. After each aerosol deposition, subjects rinsed their mouths with water, and then sat with their back to the gamma camera for two initial 2-min scans. Immediately after these scans, subjects randomized to receive induced sputum underwent a modified induced sputum procedure while seated in front of the gamma camera.

After sputum induction, subjects remained in front of the gamma camera for an additional 1.5 h. Subjects who were not randomized to receive an induced sputum after their aerosol deposition remained seated in front of the gamma camera for 2-h after aerosol deposition. Subjects returned the following day for a 24-h postretention gamma camera image. At least 48 h later, subjects returned for their second visit period and received a randomized aerosol deposition with or without induced sputum. A total of four conditions completed the study: (*1*) peripheral deposition with induced sputum; (*2*) peripheral deposition without induced sputum; (*3*) central deposition with induced sputum; and (*4*) central deposition without induced sputum.

Central (Bolus) Airway Deposition Technique

An aerosol bolus is a discrete volume of air containing particles sandwiched within an inhaled volume of particle-free air. The depth to which the bolus penetrates into the lung is determined by the volume of the bolus and the volume of air inhaled after its insertion into the air stream. An injection at the beginning of an inhalation would preferentially deliver the aerosol to the periphery, whereas inhalation of the bolus at the end of a breath tends to deliver the aerosol to the extrathoracic and conducting airways. The inhalation is generally followed by a period of breath holding to maximize particle deposition on airway surfaces. In attempting to confine the aerosol to the ADS of the lungs, the boluses are small (< 50 ml) and delivered to shallow volumetric front depths (VFD), i.e., less than 150 ml (13, 14). The VFD is an estimate of the penetration of particles into the respiratory tract and represents the volume inspired from the point when the first particles enter the mouth to the end of inhalation (14). By normalizing the VFD to an individual's ADS, the aerosol bolus may deposit more similarly in subjects of variable lung size.

On our central (bolus) airway deposition study days, a small bolus (40 ml) was delivered to shallow VFDs in the lung. Radiolabeled (^{99m}Tc) SC (50 mCi) was prepared using TechneScan Sulfur Colloid Kits (Mallinckrodt Medical, Maryland Heights, MO) (16). The binding of ^{99m}Tc to SC was always greater than 99% determined by paper chromatography. The ^{99m}Tc-SC particles in solution are submicronic with a number mean diameter of 0.22 μm and geometric standard deviation of 1.75. The solution was then placed in a modified DeVilbiss 646 jet nebulizer, which generated 6 μm mass median aerodynamic diameter (MMAD) polydisperse (when pulsed at 40 psi), i.e., aqueous particles containing the smaller suspended ^{99m}Tc-SC particles. In this way, the ^{99m}Tc-SC particles were deposited as 6-μm droplets but dissociated into 0.22-μm particles. By computer-controlled activation of the compressed air source used to disperse the nebulizer solution, the boluses were delivered to a VFD of 0.6 ADS for each subject. After inhalation of each bolus under controlled conditions (500 ml tidal volume at 125 ml/s flow), the subject held his or her breath for 5 s followed by a rapid exhalation to maximally deposit the particles on the conducting airways. Also during the bolus inhalations the relative aerosol concentration and respired volumes were measured by photometry and a pneumotachograph at the mouth to determine the VFD of each inhaled bolus. In general, inhalation of 20 to 30 boluses over a 15-min period was required to deliver sufficient activity to the lungs (approximately 15 μCi), as monitored by a single-crystal NaI detector placed at the subject's back.

Peripheral Airway Deposition Technique

To deliver particles to the peripheral, alveolar region of the lung, we nebulized radiolabeled SC solution with an Ultravent nebulizer (Mallinckrodt Medical), which produced a mean droplet size of 0.7 μm. Subjects inhaled this aerosol while matching a 1-L tidal volume, 12 breaths/minute breathing pattern displayed on an oscilloscope. The combination of this slow, deep breathing pattern and the small particle size was chosen to maximize alveolar deposition relative to that in the bronchial airways (17, 18). The subjects inhaled the radiolabeled aerosol until approximately 15 to 20 μCi were deposited in their lungs (< 2 min), again as monitored by the single-crystal NaI detector placed at their backs.

Gamma Camera Analysis

To assess the degree of central (C) versus peripheral (P) airway deposition within the lung for all four study days in each subject, we calculated a C/P ratio of ^{99m}Tc activity, normalized to the xenon-133 equilibrium scan, on the initial deposition scan after radiolabeled aerosol inhalation (12, 19). This normalization was done to account for the difference in relative lung areas and thickness between the central and pe-

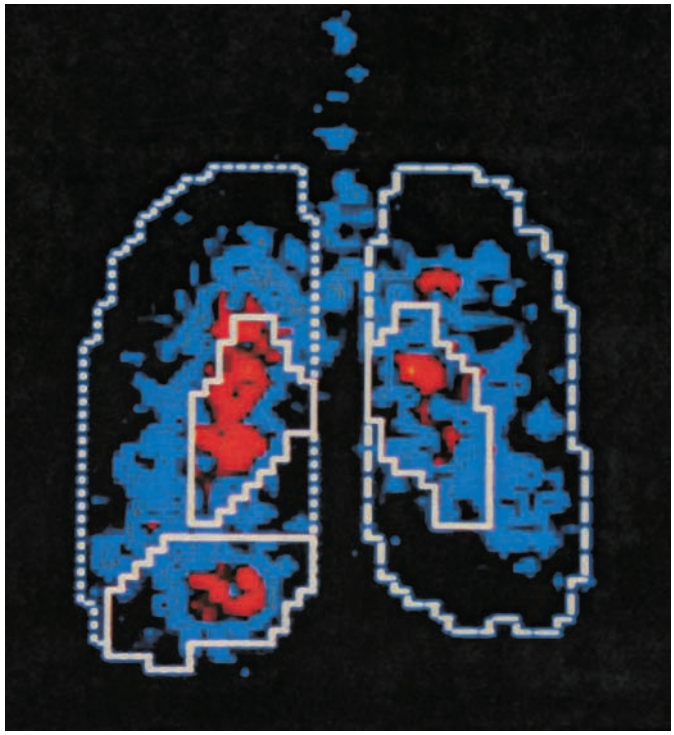

Figure 1. Gamma camera image of the whole lung (left and right) 4 min after a central airway deposition of radiolabeled ^{99m}Tc-SC particles for one subject. *Dotted lines* indicate the outline of the right and left lung based on the xenon equilibrium scan. The central airways region of interest (ROI) (*solid line*) is shown for both lungs (area = 25% of the whole lung ROI). Greater deposited activity is shown in *red*. The ROI for the stomach is shown in the bottom quadrant of the left lung to delineate and subtract stomach activity.

ripheral regions. Whereas both the central and peripheral regions (Figure 1) overlay alveoli and small airways, the central region also incorporates large, bronchial airways not present in the peripheral region. Thus, increases in C/P to values greater than 1.0 reflect increased large airway deposition. 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 (Figure 1, lower left region). This should have had no effect on our C/P comparisons because the same regions were used for both central and peripheral deposition days and the C/P was normalized to associated lung volumes (^{133}Xe equilibrium) in each case. A rectangular region bordering the right and left lung (defined by the ^{133}Xe equilibrium scan) was used to determine, by computer analysis, the whole lung clearance as a percentage of the initial counts (background-corrected and decay-corrected) over the gamma camera scanning period of 2 h and at 24 h. Again, care was taken to ensure that the activity in the stomach was excluded from the left lung region.

Induced Sputum

Sputum induction and collection. Induced sputum samples were collected and measured for radioactivity at 40 min after aerosol deposition. We followed the method published by Pin and coworkers (20) with some modifications. In brief, forced expiratory volume in one second (FEV_1) and vital capacity (VC) were measured before saline inhalation to determine baseline values. The FEV_1 values that matched a 20% fall from baseline were determined and recorded. An ultrasonic nebulizer was filled with 30 ml of 5% hypertonic saline (NaCl) for the first of two 12-min inhalations. During each inhalation period, the subject was encouraged to come off the mouthpiece at any time to cough if a sputum sample from the lower airways (i.e., not from the back of the throat) was ready for expectoration. A separate sterile container designated as waste was used to collect saliva–salt water that collected in the mouth and throat during the inhalation periods. After the 12-min inhalation period and before expectoration, subjects underwent a three-step cleansing procedure: (*1*) Gargle and rinse the mouth with water. (*2*) Scrape and clear the back of the throat (to avoid the inclusion of nonairway fluid samples), and then expectorate this into the sink. (*3*) Blow one's nose. Next the subjects delivered a chesty cough and expectorated the secretions into a sterile specimen jar, which was capped and placed on ice. Each subject coughed as many times as he or she felt necessary in order for airway secretions to be expectorated. This was typically 5 to 7 cough attempts. A postinduction measurement of FEV_1 was then performed. Provided the FEV_1 decreased by less than 20% from the baseline value, the subjects repeated the second 12-min inhalation period with 5% saline followed by the cleansing procedure and cough attempt.

Sputum Analysis

Immediately after sputum induction, the expectorated sample and waste container were weighed and taken to a scintillation counter (Nuclear Data Sodium Iodide [NaI] Scintillation Counter; Smyrna, GA) to determine the level of radioactivity (counts) present in each. The sputum sample was transferred to a sterile Petri dish and its characteristics macroscopically (or microscopically if necessary with an inverted microscope) recorded in terms of color, consistency, and degree of salivary contamination. The sputum plugs were manually separated from surrounding saliva with sterile forceps, selected, and transferred to a preweighed test tube. Radioactivity was measured on the selected sputum sample and recorded. The radioactive counts associated with the sputum sample were normalized to the amount of activity deposited in the lung for each day. The sample was then weighed and a volume of 0.1% dithiothreitol (DTT) equal to 4× the sample weight was added to the sample. Separate experiments determined that DTT had no effect on the integrity of the ^{99m}Tc label to remain affixed to the SC particles. The sample was gently aspirated with a pipette and agitated (bench rocked) for 15 min. A volume of Dulbecco's phosphate-buffered saline (D-PBS) equal to 4× the sample weight was then added to the sample, and the sample was bench rocked for 5 min.

The sample was then filtered through a 48- to 52-μm diameter nylon mesh and the weight of the cell suspension was recorded. After this, the sample was centrifuged (1,000 rpm, 5 min) and the supernatant collected into a separate tube. A separate experiment also showed that this level of centrifugation was not capable of spinning down the "free" SC particles in solution. The level of radioactivity was measured in the cell pellet and supernatant. Next, a Neubauer hemocytometer and trypan blue exclusion staining (1:1 dilution of sample:trypan blue) were used to determine the total cell count (excluding squamous epithelial cells) and cell viability.

Normalization of Sputum Radioactivity to Deposited Radioactivity

The radioactivity measured in the sputum samples by the scintillation detector was also dependent on the total amount of activity deposited in the lung for either the central or peripheral depositions. It was therefore necessary to normalize these sputum counts in order to make both intrasubject and intersubject comparisons. This normalization was accomplished as follows:

$$\text{NRC (normalized radioactive counts)} = \text{Measured counts} \times \frac{(\text{whole lung gamma camera counts for specific subject and study day})}{(\text{average whole lung gamma counts for all subjects on both central and peripheral sputum induction study days})}$$

The whole lung gamma camera counts were determined from the initial deposition image (i.e., Figure 1) (before beginning the sputum induction procedure) and the rectangular regions bordering the left and right lungs. For each subject and study day, the measured counts were first background-corrected and decay-corrected (^{99m}Tc has a 6-h half-life) to the time of the initial gamma camera deposition image.

Statistical Methods

Statistically significant differences between multiple study end points were assessed using a repeated-measures one-way analysis of variance

TABLE 1. ANTHROPOMETRIC DATA

Sex	Age (*yr*)	Height (*cm*)	Weight (*kg*)	Smoker	FEV_1 (*L*)	FVC (*L*)	% Pred FEV_1	% Pred FVC	ADS (*ml*)
F	34	160	64	No	2.63	3.55	92	106	150
F	26	173	62	No	3.42	4.19	100	104	150
M	50	190	105	No	5.40	6.73	116	117	270
F	52	157	82	No	2.36	2.67	116	108	130
M	22	191	77	No	4.74	6.15	91	102	170
M	44	196	93	No	4.07	6.08	90	106	180
M	20	173	84	No	3.30	4.07	80	85	170
F	21	168	67	No	3.46	3.87	121	117	130
F	23	163	73	No	3.11	3.42	115	110	160
F	20	168	61	No	3.12	3.64	108	109	130
Average	31.2	173.9	76.8		3.56	4.43	103	106	160
SEM	4.0	13.7	4.6		1.12	1.39	4	3	10

Definition of abbreviation: ADS = anatomic dead space.

TABLE 2. TOTAL AND DIFFERENTIAL CELL COUNTS FROM INDUCED SPUTUM*

Aerosol Deposition	Total Sputum Sample (*mg*)	Total Selected Sputum Sample (*mg*)	Total Cells (10^6)	% PMN†	% Mac†	% Lym†	Cell Viability (%)
Central	7,272 ± 736	3,446 ± 448	3.653 ± 0.5	23.3 ± 6.6	75.7 ± 7.5	1.14 ± 0.5	73.44 ± 3.9
Peripheral	7,555 ± 1,020	2,663 ± 434	3.819 ± 1.0	15.5 ± 2.39	83.5 ± 12.0	1.28 ± 0.89	60.0 ± 5.9

Definition of abbreviations: Lym = lymphocytes; Mac = macrophages; PMN = polymorphonuclear neutrophils.
* Values are mean ± SEM.
† Differential cell counts are % nucleated cells excluding squamous epithelial cells. Mean % squamous epithelial cells were 10.1% (central) and 16.5% (peripheral).

(ANOVA) followed by Tukey's multiple comparison *post hoc* analysis. Analysis between two study end points was assessed using Student's *t* test (two-tailed). Welche's correction was applied when equal variances could not be assumed or were found to be significantly different. A p value of < 0.05 was considered statistically significant.

RESULTS

Table 1 shows anthropometric data for all subjects (4 male, 6 female). Each subject met the spirometric inclusion criteria of $\geq 80\%$ of predicted baseline values for FEV_1 and FVC. Mean (SEM) group values for percentage of predicted FEV_1 and FVC were 103 and 106%, respectively.

Table 2 shows the mean (SEM) total and differential cell count data from induced sputum samples collected after central and peripheral aerosol depositions. No significant differences ($p < 0.05$) were observed in total and differential cell counts between central and peripheral aerosol depositions. Satisfactory sputum samples were obtained from all subjects on all occasions (i.e., each selected sputum sample from all subjects on all occasions contained greater than 1,000 mg of selected sample).

Figure 2 shows the mean ± SEM number of NRC recovered in the total expectorated sputum sample, the manually selected portion of the total expectorated sample, and the nonprocessed waste (saliva generated from the induction process) for the central deposition. A minimal number of NRC was present in the waste component (i.e., waste/total = 4.8%), reflecting a negligible contribution of counts owing to deposition in the mouth and oropharynx during the deposition procedure. In contrast, a high percentage of NRC was recovered in the selected portion of the sputum sample (i.e., selected/total = 86%), indicating that the majority of radiolabeled particles were associated with sputum plugs.

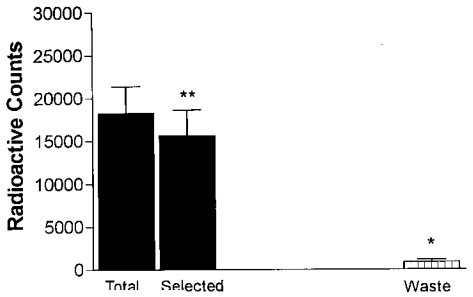

Figure 2. Mean (SEM) NRC present in the total expectorated sputum, selected sputum, and nonprocessed waste after a central airways deposition in healthy individuals (n = 10). A minimal amount of radioactivity was present in the waste component (*indicates < 5% of the total) compared with the selected component (**indicates 86% of the total). *Total* = total expectorated sputum sample; *Selected* = manually selected portion of the sputum sample; *Waste* = nonprocessed saliva generated from the induction process; *indicates < 5% of the total; **indicates 86% of the total.

Figure 3 shows the mean ± SEM NRC recovered in the total selected sputum sample, the processed cell pellet, and the supernatant in 10 subjects. The mean NRC was significantly higher ($p = 0.001$) in the selected sputum, cell pellet, and supernatant samples after a central deposition versus a peripheral deposition. The difference in NRC between the total expectorated sample and the sum of the cell pellet and supernatant was caused by retained radiolabeled particles on the nylon-mesh filter paper as a result of the sputum processing procedure.

Figure 4A shows the clearance kinetics of radiolabeled particles from the whole lung following sputum induction at 40 min, 120 min, and 24 h after central and peripheral aerosol deposition. Significantly more radiolabeled particles were cleared from the whole lung at 40 min, 120 min, and 24 h after central versus peripheral airways deposition ($p = 0.0001$). Figure 4B shows the C/P ratio for the central and peripheral airway depositions. A significantly higher C/P ratio was observed with the central deposition relative to the peripheral deposition ($p = 0.0005$, paired *t* test).

Figure 5A demonstrates the effect of induced sputum on clearance kinetics for the central deposition. Initial C/P ratios were not different between the control and induced sputum study days (C/P = 1.93 ± 0.27 versus 1.91 ± 0.39). Compared with the control condition where no induced sputum was performed, induced sputum significantly enhanced ($p = 0.001$) the clearance of radiolabeled particles from the whole lung at both 40 and 120 min after central deposition. There was no observed effect of sputum induction on clearance at 24 h after deposition. Figure 5B shows the effect of induced sputum on clearance after a peripheral deposition. Again, there was no difference in initial C/P ratios for control versus sputum induction study days (C/P = 1.19 ± 0.16 versus 1.22 ± 0.17). Unlike the central deposition, no significant differences between sputum and control were observed at the 40-min and 120-min postdeposition time points.

DISCUSSION

The widespread use and growing popularity of induced sputum as a means of examining airways inflammation in human volunteers has made it important to confirm experimentally the location from which the sputum sample derives. Several studies already suggest that induced sputum selectively samples the central airways (1–5). These studies performed comparative leukocyte analyses on induced sputum, BAL, and bronchial secretions and showed greater neutrophil proportions in both sputum and bronchial samples compared with BAL. Although these data provide useful information to support the premise that sputum is an airways phenomenon, they do not necessarily test this hypothesis.

In this study, we used a novel radiolabeled aerosol bolus delivery system that yielded more quantifiable data to show that induced sputum selectively samples the central airways with little or no contribution from the peripheral airways. The success of the bolus technique to deposit relatively more particles in the central versus peripheral airways was evident in two

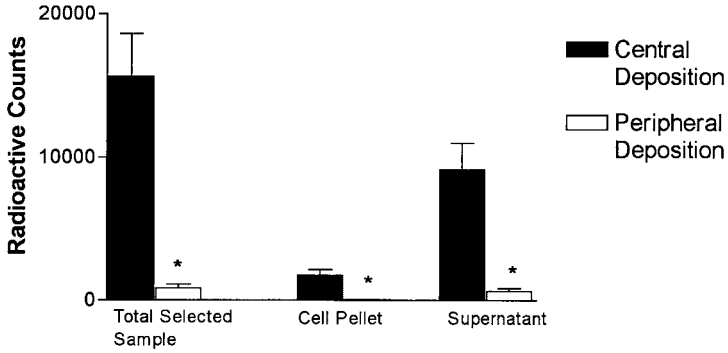

Figure 3. Mean (SEM) NRC recovered in the total selected sputum sample, cell pellet, and supernatant fraction immediately after deposition of radiolabeled aerosol in central versus peripheral airways in healthy individuals (n = 10). Significantly more radioactivity was present in all sample portions after the central deposition (*filled bar*) versus peripheral (*open bar*) deposition (*p = 0.001). *Significantly different from central deposition (p = 0.001).

ways. First, the C/P ratio was greater than 1 and significantly increased for the central versus peripheral deposition, implying greater deposition in all the bronchial airways, because deposition at the level of generations 1 to 3 will not likely occur independent of enhanced deposition throughout the rest of the conducting airways (generations 4 to 16). Second, we found that with the central deposition maneuver, approximately 60% of the particles cleared by 24 h (with or without sputum induction, Figure 5A), whereas with the peripheral deposition maneuver we only had approximately 20% clearance of particles through 24 h. From our own work (12), it is reasonable to assume that percentage of clearance through 24 h is an estimate of particles initially depositing in the conducting airways. Consequently, this represents a 3-fold greater particle deposition in conducting airways for the central versus peripheral maneuver. In addition to successfully applying the bolus technique in this study, we also considered whether any potential biases occurred in the concentration of deposited aerosol between peripheral and central airways owing to the possible differences that may be present in airways surface liquid volume. Based on estimates from the literature of airways surface liquid volume depth (10 to 30 μm) (21) and airways surface area (6,300 cm^2) (22), we calculated the airways surface liquid volume to be 6.3 ml compared with a morphologically estimated alveolar fluid volume of 7.15 ml (23, 24). These limited data suggest that volumes of airways surface liquid may not differ that much between airway and alveolar regions. Consequently, there should be minimal bias associated with variable airways surface liquid volumes in central and peripheral regions.

When we compared the level of radioactivity recovered in the selected sputum sample after a central versus peripheral airways deposition, we observed a marked increase in the number of NRC recovered in all portions of the selected sample (total, cell pellet, supernatant) after a central airways deposition. The NRC in the selected portion represented 86% of the total sample, whereas its weight was less than 50% of the total. This indicates that particles were concentrated in the selected portion of the

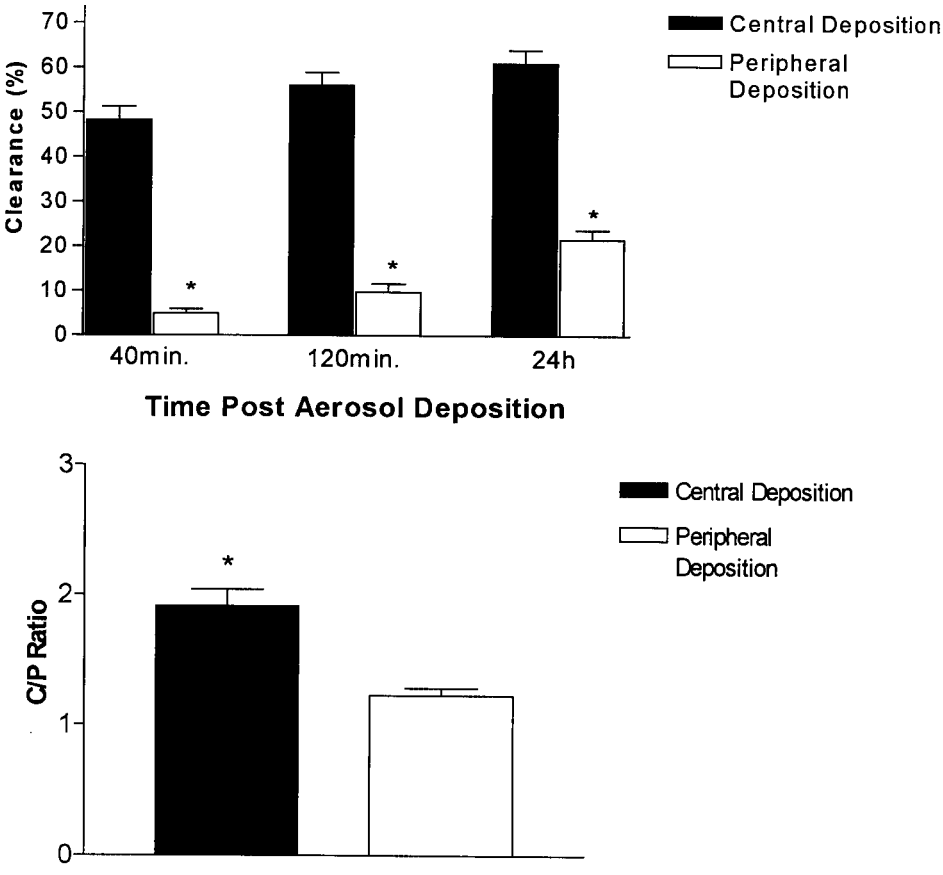

Figure 4. (*A*) Mean (SEM) whole lung clearance of radiolabeled particles with induced sputum after central and peripheral airways deposition in healthy individuals (n = 10). Significantly greater clearance was observed at 40 min, 120 min, and 24 h for sputum induction after a central (*dark bar*) and peripheral (*clear bar*) airways deposition (*p = 0.0001). (*B*) Mean (SEM) C/P ratios for the central and peripheral airway deposition in healthy individuals (n = 10). Significantly greater C/P ratios were observed after the central (*filled bar*) versus peripheral (*open bar*) airways deposition (*p = 0.0005).

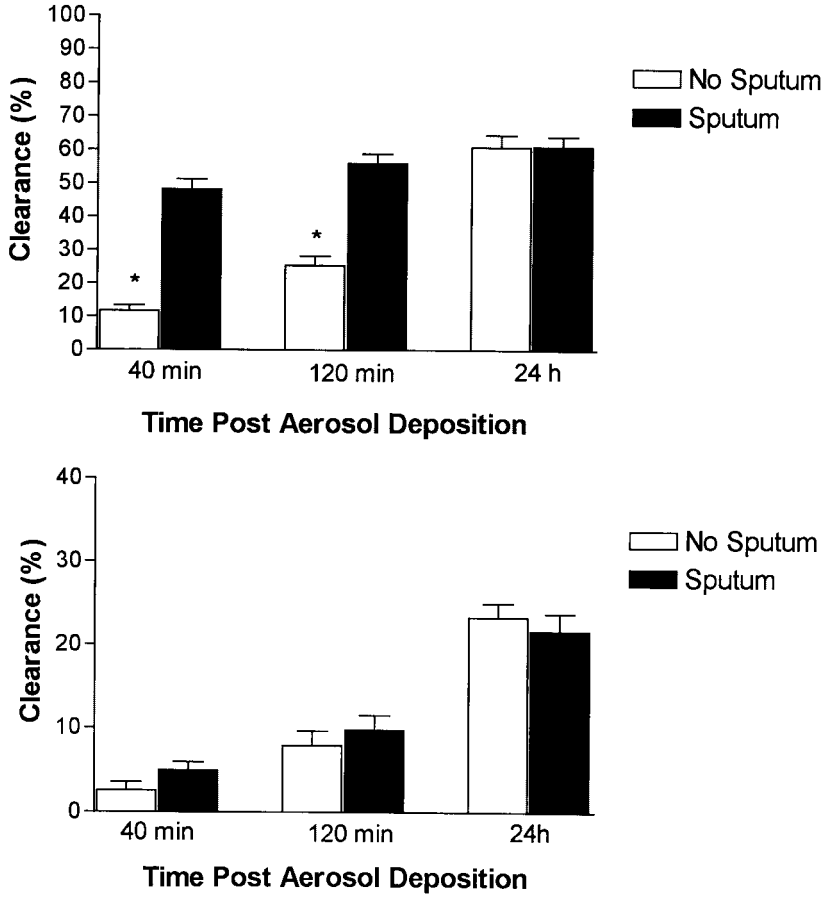

Figure 5. (*A*) Effect of induced sputum on mean (SEM) whole lung clearance of radiolabeled particles after a central deposition in healthy individuals (n = 10). Significantly greater clearance was observed at the 40-min and 120-min time points after a central airways deposition with induced sputum (*filled bar*) versus without induced sputum (*open bar*) (*p = 0.0001). (*B*) Effect of induced sputum on mean (SEM) whole lung clearance of radiolabeled particles after a peripheral deposition in healthy subjects (n = 10). There was no significant ($p > 0.05$) difference in clearance with induced sputum versus without induced sputum at 40 min, 120 min, and 24 h after a peripheral airways deposition.

sputum sample and not simply due to selecting 86% of the total sample weight. Furthermore, there were minimal (yet detectable) levels of NRC present in the sputum samples after the peripheral deposition. If induced sputum did in fact sample the peripheral airways to any significant degree, we clearly would not have observed as great a magnitude difference in particle recovery between the central and peripheral airway depositions. The detection of some radioactive counts after the peripheral deposition reflects the fact that a minimal amount of aerosol was deposited in the bronchial airways during the peripheral deposition condition. This is supported by the fact that the bulk of radioactive counts from the total sputum samples (either on central or peripheral study days) were associated with the selected "plug" sample, and within that, most of the particles were associated with the supernatant compared with the cells. Because the supernatant is likely to contain the mucous gel portion of airway secretions, and because mucins primarily derive from glandular and goblet cell secretions of conducting airways, these data provide further evidence that the total sputum samples after central and peripheral deposition are derived from the same conducting airways. Moreover, because we observed a significantly greater C/P ratio and a greater percent clearance at 24 h for the central versus peripheral deposition, we were assured that the bolus aerosol deposition more selectively targeted the central airways relative to the peripheral airways, and hence established valid central and peripheral deposition conditions.

The measurement and comparison of clearance kinetics after a central and peripheral airways deposition also provided good evidence of selective central airways sampling by induced sputum (Figure 4A). Previous studies have shown that inhalation of hypertonic saline, or cough alone, can enhance clearance of deposited particles from the lung when compared with clearance without hypertonic saline or clearance without cough. However, these previous studies (25, 26) did not attempt to distinguish between centrally (airway) and peripherally (alveolar) deposited particles when assessing the effects of hypertonic saline or cough. After sputum induction, we observed 50% clearance at 40 min for a central deposition, compared with a negligible 5.0% clearance for a peripheral deposition. This is consistent with previous reports that little or no aerosol is expected to be cleared at 40 min after a peripheral airway deposition (18, 19). Because induced sputum employs the combination of both hypertonic saline inhalation and cough, it may not have been surprising that we demonstrated greatly enhanced clearance, especially for our central deposition pattern, compared with the no sputum control condition, considering that cough is most effective in the large conducting airways, where shear rates are highest (greatest air velocities) and the depth of airway surface fluid is likely greatest (27). Finally, as further evidence that the sputum induction technique does not sample the deep lung, the 24-h retention (i.e., 100 − % 24-h clearance, Figures 5A and 5B) was unchanged for both central and peripheral depositions between the sputum and no sputum induction study days. This percentage of particles retained at 24 h is a reflection of those particles depositing deepest (most alveolar) in the lung (12, 18, 19). These data further confirm our hypothesis that induced sputum samples derived from the central airways, not the alveolar regions of the lung.

For the central deposition sample, the average ratio of activity in the cell pellet (i.e., cell-associated particle ratio) versus the sum of the cell pellet plus supernatant was 12 ± 3%, if we considered only those individuals for whom we recovered more than 80% of total sputum activity in the cell pellet plus supernatant. Similar radiolabeled aerosol studies performed in animal models (hamster macrophages) show a higher cell-asso-

ciated particle ratio (32 to 34%) immediately after deposition (28, 29). Experimentally, preferential filtering of cell-associated particles during sputum processing in our experiments may partially explain this difference, but several other reasons might also explain the greater uptake of particles in the animal studies. These include species differences, a larger particle size used in the animal studies (6-μm polystyrene particle versus 0.2-μm SC particle), as well as a higher delivered dose of particle to the animals' airways. A greater particle size and higher delivered dose of particles to the hamsters' airways may have stimulated active recruitment of monocytes and monocyte-like macrophages to the site of deposition in the airways. Because newly recruited monocytes and monocyte-like macrophages are more phagocytic compared with macrophages with a longer residence time in the airways (1), a large proportion of the macrophage pool in the hamster airways may have possessed increased phagocytic capacity. In our study, it was unlikely that the small particle load delivered in 20 to 30 boluses stimulated any significant recruitment of young phagocytes into the airways from the peripheral blood. Consequently, a comparatively smaller proportion of highly phagocytic macrophages may have interacted with the deposited particles in our subjects. It is interesting to speculate whether the airway macrophages in our subjects required a longer incubation time (before sputum induction) to optimally interact with the deposited aerosol to produce a greater cell-associated particle ratio, possibly approaching the percentages observed in the animal studies. Time course studies are currently under way in our laboratory to investigate this intriguing question.

Conclusions

With the use of a radiolabeled aerosol bolus inhalation technique, this study directly tested and confirmed the hypothesis that induced sputum samples derive from the central airways with little or no contribution from the peripheral airways. In addition, the combination of hypertonic saline and spontaneous cough did not enhance clearance of particles from the peripheral alveolar spaces. Finally, preliminary data suggest minimal interaction between airway macrophages and deposited particles immediately after deposition.

References

1. Alexis N, Soukup J, Ghio A, Becker S. Sputum phagocytes from healthy individuals are functional and activated: a flow cytometric comparison with cells in bronchoalveolar lavage and peripheral blood. *Clin Immunol* 2000;97:21–32.
2. in't Veen JC, Grootendorst DC, Bel EH, Smits HH, Van Der Keur M, Sterk PJ, Hiemstra PS. CD11b and L-selectin expression on eosinophils and neutrophils in blood and induced sputum of patients with asthma compared with normal subjects. *Clin Exp Allergy* 1998;28:606–615.
3. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995;95:843–852.
4. Alexis N, Urch B, Tarlo S, Corey P, Pengelly D, O'Byrne P, Silverman F. Cyclooxygenase metabolites play a different role in ozone-induced pulmonary function decline in asthmatics compared to normals. *Inhal Toxicol* 2000;12:1205–1224.
5. Moodley YP, Krishnan V, Lalloo UG. Neutrophils in induced sputum arise from the central airways. *Eur Respir J* 2000;15:36–40.
6. Keatings VM, Evans DJ, O'Connor BJ, Barnes PJ. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washing, and bronchoalveolar lavage fluid. *Thorax* 1997;52:372–374.
7. Grootendorst CD, Sont JK, Willems LNA, Kluin-Nelemans JC, Van Krieken JH, Veselic-Charvat M, Sterk PJ. Comparison of inflammatory cell counts in asthma: induced sputum versus bronchoalveolar lavage and bronchial biopsies. *Clin Exp Allergy* 1997;27:769–779.
8. Maestrelli P, Saetta M, Di Stefanno A, Calcagni PG, Turato G, Ruggieri MP, Roggeri A, Mapp CE, Fabbri LM. Comparison of leukocyte counts in sputum, bronchial biopsies, and bronchoalveolar lavage. *Am J Respir Crit Care Med* 1995;152:1926–1931.
9. Fahy JV, Wong H, Liu J, Boushey HA. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. *Am J Respir Crit Care Med* 1995;152:53–58.
10. Clarke SE, Pavia D. Deposition and clearance. In: Murray JF, Nadel JA, editors. Textbook of respiratory medicine. Philadelphia: W.B. Saunders; 1988. p. 313–332.
11. Kim CS, Hu SC, DeWitt P, Gerrity TR. Assessment of regional deposition of inhaled particles in human lungs by serial bolus delivery method. *J Appl Physiol* 1996;81:2203–2213.
12. Bennett WD, Scheuch G, Zeman KL, Brown JS, Kim C, Heyder J, Stahlhofen W. Bronchial airway deposition and retention of particles in inhaled boli: effect of anatomic dead space. *J Appl Physiol* 1998;85:685–694.
13. Scheuch G, Philipson K, Falk R, Anderson M, Svartengren M, Stahlhofen W, Camner P. Retention of particles inhaled in boli with and without induced bronchoconstriction. *Exp Lung Res* 1995;21:901–916.
14. Scheuch G, Stahlhofen W, Heyder J. An approach to deposition and clearance measurements in human airways. *J Aerosol Med* 1996;9:35–41.
15. Fowler WS. Lung function studies: II. The respiratory dead space. *Am J Physiol* 1948;134:405–416.
16. Lay JC, Berry CR, Kim CS, Bennett WD. Retention of insoluble particles following local intrabronchial deposition in dogs. *J Appl Physiol* 1995;79:1921–1929.
17. Valberg PA, Brain JD, Sneddon SL, LeMott SR. Breathing patterns influence aerosol deposition sites in excised dog lungs. *J Appl Physiol* 1982;53:824–837.
18. Stahlhofen W, Gebhart J, Heyder J. Experimental determination of regional deposition of aerosol particles in the human respiratory tract. *Am Ind Hyg Assoc J* 1980;41:385–398.
19. Ilowite J, Smaldone GC, Perry R, Bennett WD, Foster WM. The relationship between tracheobronchial clearance rates and sites of initial deposition in man. *Arch Env Health* 1989;44:267–273.
20. Pin I, Freitag AP, O'Byrne P, Girgis-Gabardo A, Watson RM, Dolovich J, Denburg JA, Hargreave FE. Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. *Am Rev Respir Dis* 1992;145:1265–1269.
21. Widdicombe JH, Widdicombe JG. Regulation of human airway surface liquid. *Respir Physiol* 1995;99:3–12.
22. Weibel ER. Morphology of the lung. New York: Academic Press; 1963.
23. Gehr P, Bachofen M, Weibel ER. The normal human lung: ultrastructure and morphometric estimation of diffusion capacity. *Respir Physiol* 1978;32:121–140.
24. Gil J, Weibel ER. Improvements in demonstration of lining layer of lung alveoli by electron microscopy. *Respir Physiol* 1969;8:13–36.
25. Daviskas E, Anderson SD, Gonda I, Eberl S, Meikle S, Seale JP, Bautovich GJ. Inhalation of hypertonic saline aerosol enhances mucociliary clearance in asthmatic and healthy subjects. *Eur Respir J* 1996;9:725–732.
26. Bennett WD, Foster WM, Chapman WF. Cough-enhanced mucus clearance in the normal lung. *J Appl Physiol* 1990;69:1670–1675.
27. Leith DE, Butler JP, Sneddon SL, Brain JD. Cough. In: AP Fishman, editor. Handbook of physiology, Section 3, The respiratory system, Vol. 3 Mechanics of breathing, Part 1. Bethesda, MD: American Physiological Society; 1986. p. 315–336.
28. Geiser M, Baumann M, Cruz-Orive LM, Hof V IM, Waber U, Gehr P. The effect of particle inhalation on macrophage number and phagocytic activity in the intrapulmonary conducting airways of hamsters. *Am J Respir Cell Mol Biol* 1994;10:594–603.
29. Geiser M, Cruz-Orive LM, Waber U, ImHof V, Gehr P. Particle retention in airways estimated by unbiased stereology and photometry. *J Aerosol Med* 1995;8:149–165.