Methodology for the measurement of mucociliary function in the mouse by scintigraphy

W. MICHAEL FOSTER,1 DIANNE M. WALTERS,2 MALINDA LONGPHRE,3 KRISTIN MACRI,2 AND LAURA M. MILLER3

1Department of Pulmonary and Critical Care Medicine, Duke University Medical Center, Durham, North Carolina 27710; 2Johns Hopkins University School of Hygiene, Baltimore, Maryland 21205; and 3Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543

Received 10 July 2000; accepted in final form 16 October 2000

Foster, W. Michael, Dianne M. Walters, Malinda Longphre, Kristin Macri, and Laura M. Miller. Methodology for the measurement of mucociliary function in the mouse by scintigraphy. J Appl Physiol 90: 1111–1118, 2001.—The objective of the study was to develop a scintigraphic method for measurement of airway mucociliary clearance in small laboratory rodents such as the mouse. Previous investigations have characterized the secretory cell types present in the mouse airway, but analysis of the mucus transport system has been limited to in vitro examination of tissue explants or invasive in vivo measures of a single airway, the trachea. Three methods were used to deposit insoluble, radioisotopic colloidal particles: oropharyngeal aspiration, intratracheal instillation, and nose-only aerosol inhalation. The initial distribution of particles within the lower respiratory tract was visualized by γ-camera, and clearance of particles was followed intermittently over 6 h and at the conclusion, 24 h postdelivery. Subsets of mice underwent lavage for evidence of tissue inflammation, and others were restudied for reproducibility of the methods. The aspiration and instillation methods of delivery led to greater distributions of deposited activity within the lungs, i.e., ~60–80% of the total respiratory tract radioactivity, whereas the nose-only aerosol technique attained a distribution of 32% to the lungs. However, the aerosol technique maximized the fraction of particles that cleared the airway over a 24-h period, i.e., deposited onto airway epithelial surfaces and cleared by mucociliary function such that lung retention at 24 h averaged 57% for delivery by aerosol inhalation and ≥80% for the aspiration or intratracheal instillation techniques. Particle delivery methods did not cause lung inflammation/injury with use of inflammatory cells and chemotactant cytokines as criteria. Scintigraphy can discern particle deposition and clearance from the lower respiratory tract in the mouse, is noninvasive and reproducible, and includes the capability for restudy and lung lavage when time course or chronic treatments are being considered.

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Address for reprint requests and other correspondence: W. M. Foster, Pulmonary and Critical Care Medicine, Duke Univ. Medical Center, PO Box 2629, Durham, NC 27710.

THE MUCOUS LINING OF THE MAMMALIAN respiratory tract originates primarily from products of secretory cells interspersed among mucosal cells or located within submucosal glands. Airway mucus is continuously transported to the larynx from peripheral airways by ciliary activity of the underlying epithelium (7, 28). This mucociliary transport system, which also participates in heat and water exchange (4), is essential for the protection of the conducting airway surfaces from ambient irritants and infectious agents and for maintenance of airway patency.

A number of airway disease states have been modeled in the laboratory mouse (21, 27). The principal nonciliated cell type of the surface epithelium of the larger airways in the mouse is morphologically identical to the Clara cell of the distal airway (17), with its luminal cytoplasmic projection filled with smooth endoplasmic reticulum and irregular electron-dense granules. Because this cell type is found at every airway level in the mouse from the distal airway to the larynx and normally airway mucous and serous cells are absent, it has been suggested that the Clara cell is the primary source of respiratory mucus. Submucosal glands are an additional source of respiratory mucus in the mouse, but these are limited to the larynx and the upper part of the tracheal airway, with only a low number, or even total absence, of glands in the lower part of the trachea (5, 31).

Mucociliary function in the mouse has been studied in explanted lung tissue by use of tangentially sliced airways and transport of charcoal particles (11). This in vitro approach has exhibited good stability and reproducible mucociliary clearance responses to physiological stimuli. In vivo, the mucociliary transport velocity of a single airway, the trachea, has been investigated invasively and been found impaired in transgenic (insertional mutagenesis of murine CFTR gene) compared with control mice (31). Mucus in the transgenic mice may have been abnormally dehydrated and at greater depths overlying the tracheal epithelium. These results suggest that scintigraphy, a noninvasive approach that has been used to character-
ize mucociliary transport mechanisms in humans and large animal models (8, 14, 25), may have useful application in the mouse and permit in vivo investigation of mucociliary clearance from the entire airway. As a first step, we used a two-dimensional γ-imaging technique in mice to measure the time course of clearance of an insoluble radiotracer deposited onto the epithelial surfaces of the airway and distal lung. Radiolabeled particles were deposited in the lung by three separate methods: nose-only aerosol inhalation, oropharyngeal aspiration, and intratracheal instillation, after which clearance of the insoluble particles was dynamically followed. To our knowledge, scintigraphy has not been utilized in small rodents to quantitate airway mucociliary function, and this technique conveys certain advantages in being noninvasive and providing clearance data from the entire airway and includes the capability to restudy animal subjects when time course and/or prolonged treatment(s) are being considered.

METHODS

The study protocol conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare guidelines for the experimental use of animals and was approved by The Johns Hopkins Animal Care and Use Committee.

Mice. Five- to eight-week-old male A/J mice (Charles River Laboratories, Frederick, MD) were housed in laminar flow hoods in an environmentally controlled animal facility for the duration of the experiment. Animals were provided with rodent chow and tap water ad libitum. Mice were entered into one of three protocols. The protocols were designed to deliver insoluble radiolabeled sulfur colloid (SC) to the lower respiratory tract with one of three delivery methods. After deposition, clearance of the labeled colloid from the lungs was monitored for a 6-h period, and a final measurement of particle retention was assessed ~24 h postdeposition. To assess whether the direct delivery or aerosol inhalation methods of deposition led to tissue inflammation, subsets of mice following the 24-h image were killed, and the lungs were lavaged to obtain bronchoalveolar fluid for analysis (see Bronchoalveolar lavage).

Preparation of radiolabeled colloid and imaging method. The radiolabeled tracer that was used to gauge distribution of delivered activity and the subsequent course of clearance from the airway by mucociliary transport was 99mTc-technetium-labeled SC (99mTc-SC). 99mTc has a half-life of 6 h, and thus it is useful for measures in time of up to ~30 h when counting devices with the sensitivity of a γ-camera are used. The labeled colloid solution was freshly prepared according to the manufacturer’s (Cis, Bedford, MA) instructions, and is nonpyrogenic, isotonic, and at a neutral pH. Preparation of 99mTc-SC from commercial kits with the thiosulfate method is reported to produce polydisperse submicrometer particles (14). We have previously assayed for unbound 99mTc with silica gel media and thin-layer chromatography to verify the labeling procedures (2, 25). Immediately after deposition of the 99mTc-SC, the initial distribution and subsequent clearance of the labeled SC were measured with the mice prone and were imaged from the dorsal aspect by a γ-camera. The camera was set with an 18% window around the peak energy of 99mTc and was shielded with a pinhole collimator. Pinhole collimation provided the adequate spatial resolution necessary for distinguishing location and retention of radioactivity within the lung and upper respiratory tract of the mouse. 99mTc-SC clearance from the lung was then measured over a 6-h period (reimaging 4–5 times) and at ~24 h postdelivery to assess residual retention of 99mTc-SC. On the basis of the activity (~15–60 μCi of 99mTc) deposited, the lungs were readily visualized, and high quality, analyzable images were acquired with short imaging times (i.e., initially at 40–120 s as activity cleared and decayed and at 10 min for the final image at ~24 h). In several pilot studies, the radioactivity visualized in vivo within the respiratory tract (oropharynx, trachea, and lungs) and extrapulmonary tissues (esophagus and stomach) was confirmed at necropsy 1–6 h and 24 h after deposition of 99mTc-SC. To quantify the clearance of 99mTc-SC, lung images were stored on a computer, and cursor manipulation was used to identify a lung region of interest that excluded the tracheal airway and upper respiratory tract. Activity time plots for the lung region were constructed after background and isotopic decay correction of the retention images.

Nose-only aerosol inhalation protocol. Mice (n = 18) in a conscious state were placed into 50-ml volume, conical tubes with the tips trimmed off to allow the rostrums of the mice to protrude from the ends of the tubes. Mice were then attached two at a time to a common Lucite aerosol chamber (15 × 12.5 × 12.5 cm) for nose-only exposure to a 99mTc-SC aerosol. The labeled SC aerosol was generated by jet nebulization (SyteVent, Synaco) and entrained by the airflow supply serving the aerosol chamber. The aerosol aerosol at inhalation had a mass mean diameter of 0.65 μm (σg of 1.66) that was expected to increase in size after being inhaled into the conditioned environment of the respiratory tract. Aerosol exposures lasted for ~7–9 min, after which the mice were lightly anesthetized with vaporized methoxyflurane (Mefofane, Schering-Plough Animal Products, Union, NJ). After induction of anesthesia, the mice were placed in a prone position and imaged from the dorsal aspect by a γ-camera. Mice regained consciousness in ~6–8 min but were periodically reimagened over 6 h and at ~24 h after inhalation to assess residual lung retention of 99mTc-SC. For the repeat images, the mice were lightly anesthetized each time with metofane vapor, and, at the 24-h endpoint, which required a longer imaging period, mice were anesthetized with an intraperitoneal injection of ketamine and xylazine (45 and 8 mg/kg, respectively).

Oropharyngeal aspiration protocol. Mice (n = 18) were anesthetized by an intraperitoneal injection of ketamine and xylazine (45 and 8 mg/kg, respectively) and suspended by their upper incisors from a rubber band on a 60° incline board. The tongue was gently extended, and a liquid volume (25 or 50 μl) of 99mTc-SC was delivered into the distal part of the oropharynx. With the tongue extended, the animal was unable to swallow, and the liquid volume was aspirated into the lower respiratory tract. Animals were immediately imaged but were permitted to recover and were then reimaged periodically for an additional 6 h and at ~24 h after delivery by means of positioning and being given anesthesia as described in Nose-only aerosol inhalation protocol.

Intratracheal instillation protocol. Mice (n = 12) were anesthetized by intraperitoneal injection of ketamine and xylazine (45 and 8 mg/kg, respectively) and then intubated. For intubation, the mice were suspended by their upper incisors from a rubber band on a 60° incline board. The trachea was transilluminated below the vocal cords to allow visualization of the trachea through the oral cavity. With the lower jaw held open and the tongue held out, a 2-cm length of PE-50 (Johnson and Johnson Medical, Arlington, TX) tubing with beveled tip (attached to a 20-gauge needle hub) was
gently inserted into the tracheal opening, and liquid volumes (~40 μl) of 99mTc-SC were instilled. Animals were immediately imaged, permitted to recover, and reimaged and anesthetized as described in the preceding two sections.

**Bronchoalveolar lavage.** To assess whether delivery of 99mTc-SC by the direct deposition or aerosol inhalation techniques resulted in any degree of lung inflammation, we performed bronchoalveolar lavage to monitor for the presence of inflammatory cells and mediators. Immediately after acquisition of the 24-h residual image, anesthetized mice from the nose-only inhalation protocol (n = 6) and the oropharyngeal aspiration protocol (n = 6) were killed by exsanguination through an abdominal incision and sectioning of the inferior vena cava. The chest and extrathoracic airway were surgically opened, and the tracheal airway was dissected free and cannulated. The lavage technique was modified from the methods developed by Walters et al. (25). An 18-gauge intravenous catheter (PE-90, Jelco-W, Johnson and Johnson Medical) was introduced into the lower trachea, and the lungs were lavaged with chilled Hanks’ balanced salt solution without calcium or magnesium (Biofluids, Rockville, MD). A single 1.0-ml volume of the Hanks’ solution was introduced into the tracheal catheter and slowly instilled by syringe into the lower respiratory tract, followed by gentle aspiration back into the syringe. This lavage technique was repeated two additional times with the same 1.0 ml. Recovered lavage fluid was centrifuged (300 g for 8 min), and the supernatant was removed and stored at −80°C for later measurements of cytokine protein [murine α-chemokine (mKC) and murine tumor necrosis factor-α (mTNF-α)] levels.

The cell pellet was resuspended in 1.0 ml of 10% fetal bovine serum in phosphate-buffered saline solution. The total number of cells was counted with a hemocytometer. Slides were prepared by cytocentrifugation (Cytospin 3, Shandon Instruments, Pittsburgh, PA) and stained with Diff-Quick (Dade Behring, Dudingen, Switzerland). Bronchoalveolar cell differential counts were determined using morphological criteria under a light microscope with evaluation of ≥500 cells/slide.

**Cytokine assays.** Lavage samples were plated in duplicate on 96-well microtiter plates (Immulon, PGC Scientific, Gaithersburg, Maryland). Wells containing no sample served as negative controls, and wells containing recombinant mTNF-α or mKC (R&D Systems, Minneapolis, MN) served as positive controls and concentration standards on each plate. mTNF-α and mKC were measured using sandwich ELISA kits (R&D Systems). TMB-peroxidase substrate solution (KPL, Gaithersburg, MD) was used for the ELISAs.

**RESULTS**

A representative image acquired by scintigraphy is presented in Fig. 1 after deposition of 99mTc-SC particles in the respiratory tract of a mouse. The 99mTc-SC activity was delivered by the oropharyngeal aspiration technique, and the image demonstrates the visualization of regional deposition by scintigraphy. Similar deposition images were obtained for the intratracheal instillation and nose-only aerosol inhalation techniques, although, after aerosol inhalation, nonsurgical tracheal deposition was present in a region identified as stomach and represented radioactivity that was swallowed into the digestive tract during exposure to the aerosol. However, stomach activity was seldom found at deposition with the direct delivery methods, i.e., only 1 of 18 animals after oropharyngeal aspiration and 2 of 11 animals after intratracheal instillation. On the basis of the scintigraphic images acquired immediately after deposition, the distributions of 99mTc-SC activity within the upper (oropharyngeal) and lower (lung) respiratory tract are presented in Fig. 2. The aspiration and instillation methods of delivery, as anticipated, provided a fractional deposition to the lungs smaller than that achieved by the aerosol inhalation technique. For the intratracheal instillation technique, on average 77 ± 7% (SE) of the respiratory tract activity was in the airways and lung, and this was comparable to the aspiration method that appeared to be volume dependent and delivered on average 62 ± 2 and 81 ± 2% to the lung, depending on the liquid volume being aspirated at 25 and 50 μl, respectively. The aerosol method delivered the least amount of radiotracer to the airways and lungs, i.e., 32 ± 2% of the deposited amount. Activity that was visualized midline in the image field could not be distinguished between esophageal or tracheal compartments and was not included in the data of Fig. 2. Esophageal/tracheal activity, however, represented a small percentage of the respiratory tract activity for each delivery method, i.e., 6, 4, 9, and 15% for the 25- and 50-μl aspirations, the intratracheal instillation, and the nose-only aerosol inhalation, respectively.

Differences in clearance of 99mTc-SC during the initial 6-h period after deposition and at the final 24-h endpoint were significant between the direct delivery...
methods, aspiration, and instillation and by the aerosol inhalational route. Figure 3 compares the temporal changes in airway retention of $^{99m}$Tc-SC after the three modes of delivery. For the aspiration and instillation modes of delivery, only a small percentage of the SC cleared from the lung region during the initial 6 h after deposition. After 24 h, $85 \pm 9\%$ of the initial lung activity was still retained for animals administered 50-$\mu$l volume by the aspiration method (although not shown in the figure, in eight animals evaluated with the 25-$\mu$l volume, $80 \pm 12\%$ was retained), and was comparable to the $80 \pm 7\%$ that remained in the lung when $^{99m}$Tc-SC was delivered by the intratracheal instillation technique, whereas, after aerosol delivery, on average, $30\%$ of the initial lung activity had been cleared from the lung region during the first 6 h and an additional $13\%$ was removed by 24 h. Thus, for the nose-only aerosol inhalation delivery method, the mean lung retention of $^{99m}$Tc-SC at the 24-h endpoint was $57 \pm 4\%$.

The differential cell data for the bronchoalveolar fluids collected at the 24-h endpoint after delivery of the $^{99m}$Tc-SC by nose-only inhalation and oropharyngeal aspiration are listed in Table 1. The concentrations of cytokines (TNF-$\alpha$ and KC chemokine exhibit pleiotropic proinflammatory and chemoattractant effects, respectively) in the lavage fluid are listed in Table 2. There was no evidence of an inflammatory process being present. The degree of cellularity and the levels of inflammatory cytokines present in the lavage fluids after either delivery method were similar to each other and comparable to cell populations and mediators that are normally obtained by lung lavage of naive control animals.
or oropharyngeal aspiration of radiolabeled colloid by aerosol inhalation or mTNF-α, murine tumor necrosis factor-α.

Eosinophils: 3
Lymphocytes: 3
Epithelial cells: 3
Neutrophils: 3
Macrophages: 3

Table 2. Details of bronchoalveolar inflammatory cytokines analyzed ~24 h after deposition of radiolabeled colloid by aerosol inhalation or oropharyngeal aspiration

<table>
<thead>
<tr>
<th>Cytokine Mediator</th>
<th>Naive</th>
<th>Aerosol</th>
<th>Oropharyngeal</th>
</tr>
</thead>
<tbody>
<tr>
<td>mKC</td>
<td>0.0</td>
<td>11.2 ± 4</td>
<td>0.0</td>
</tr>
<tr>
<td>mTNF-α</td>
<td>20.9</td>
<td>0.0</td>
<td>103.7 ± 34</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed in pg/ml; n = 6. mKC, murine chemokine KC; mTNF-α, murine tumor necrosis factor-α.

DISCUSSION

We have demonstrated in the mouse that γ-scintigraphy of the respiratory tract can be utilized to monitor deposition and subsequent clearance of insoluble particles by mucociliary function from the lower respiratory tract. Three techniques for deposition of an insoluble radiolabeled tracer were evaluated: nose-only aerosol inhalation, oropharyngeal aspiration, and intratracheal instillation. Each method of delivery deposited adequate amounts of activity for imaging the lower respiratory tract, and, with the direct methods, aspiration and instillation delivered, as expected, a higher percentage of the administered dose to the lungs than did the aerosol inhalation method. In the 24-h period after deposition, there were moderate levels of clearance for the direct delivery methods, i.e., 15–20%, whereas for the aerosol method, 43% of the lung dose was cleared by the 24-h endpoint.

Although γ-scintigraphy has been used to evaluate regional deposition and mucociliary clearance of the tracheobronchial airways of humans and large laboratory animal models, we are not aware of its application to the laboratory mouse for the study of particle dosimetry and clearance. Whole body scintigraphy of the mouse has proved informative for other applications, e.g., in vivo assessment of virally induced immunodeficiency (21). Mice are highly favored as models of inflammatory airway disease and recently have been shown to display cytokine-induced phenotypes associated with a hypersecretory airway epithelium (21, 27). It has been generally accepted that insoluble particles depositing onto the epithelial surfaces of the lower respiratory tract are cleared by two phases (1, 12, 16). The initial phase thought to have clearance half-times between 3 and 12 h and identified as tracheobronchial concludes by convention after 24 h. The second phase, slow compared with the tracheobronchial phase, requires several months to complete and has been identified as alveolar. The early phase is believed to represent clearance of particles that have deposited onto the ciliated surfaces of the conducting airways and that, if insoluble, are removed by mucociliary activity (7, 28).

The differences in clearance of lung activity that we observed for the aspiration and instilled-delivery modes compared with the aerosol inhalation method likely are due to differences in the major sites of deposition of the insoluble 99mTc-SC. The small fraction of the insoluble particles that cleared and the gradual rate of clearance for the aspiration and instillation delivery methods are suggestive that the particles were deposited primarily onto alveolar surfaces (13, 14). Others have shown (19), in rodent models, that, when particles are delivered directly to the lung, e.g., intratracheal administration, the distribution of deposition is very nonhomogeneous, with few or no particles being peripheral and the bulk of the material within pulmonary compartments having short path lengths from a major airway. With the aerosol method of delivery, a larger fraction of the particles cleared over 24 h, i.e., deposited onto surfaces cleared by mucociliary function and at a high rate of removal, at least over the initial 2 h of clearance. For the inhaled aerosol method, this suggests that a higher proportion of the particles is likely to deposit onto airway epithelial surfaces (7, 12, 19) and to be cleared by mucociliary mechanisms. To enhance the probability of aerosol deposition onto the airways, we favored an aerosol generation technique that would produce an aerosol at inhalation that was submicrometer in size. Previously, it had been demonstrated in mice that aerosols in this size range have a high probability of airway deposition within the lower respiratory tract.

![Activity time plots that demonstrate reproducibility of the methods. Repeatability of clearance of 99mTc-SC particles from the lung region is shown. Mean retention data (± SE) of mice (n = 4) at the indicated time points are compared for study 1 vs. study 2 (time between studies ~7 days). 99mTc-SC particles were deposited in the lungs by nose-only aerosol inhalation technique (See Fig. 3).](http://jap.physiology.org/)
respiratory site is the major reason for the fractional differences in clearance between the direct delivery methods and administration by aerosol inhalation. Although there were also differences in the doses of $^{99m}$Tc-SC administered, earlier studies have shown that, over the range of activities we utilized, the mucociliary clearance process is not affected (29, 30). In additional experiments (data not shown), when we varied the lung dose by design and doubled the activity administered by aspiration from 20 to 48 μCi of $^{99m}$Tc-SC, there was no effect on the fraction of lung activity cleared. However, at a high, effective dose of 130 μCi, lung clearance became delayed, and a smaller fraction was cleared after 24 h.

With the use of sterile conditions for radiotracer synthesis and clean delivery/deposition conditions, airway or lung tissue inflammation did not appear to result from either the direct (oropharyngeal aspiration) or aerosol delivery techniques (Tables 1 and 2). We did not find an influx of inflammatory cells or increased levels in lavage of a representative multifunctional proinflammatory cytokine, such as mTNF-α, or mKC (3, 15). Although volatile anesthetics such as halothane are known to reduce mucociliary function from the whole lung and the tracheal airway (6, 18), we do not think anesthesia was a factor or influenced our evaluations of mucociliary clearance. Volatile anesthetics can influence airway clearance if there is an extended period of the unconscious state (2–6 h) (18). After methoxyflurane, our animals were in an unconscious state only briefly (6–8 min) at each imaging period, and they completely recovered between the measurement points. General anesthetics such as barbiturates also have been shown to slow clearance 15–60 min after the induction of the unconscious state. During our delivery procedures, the aspiration and instillation mice were anesthetized only once with ketamine/xylazine for a short period (<15 min) and quickly recovered (8–10 min). If γ-scintigraphy is to be used to measure insoluble particle clearance in the mouse, it appears that deposition by aerosol inhalation is the favorable delivery technique for administering a large fraction of marker activity to the airway and assessing mucociliary defense mechanisms. Direct delivery methods (aspiration and instillation) provide an effective peripheral or alveolar fraction and a greater proportion (>70%) of the lung dose being retained after 24 h. None of the $^{99m}$Tc-SC delivery techniques led acutely to inflammation of pulmonary tissues. To further establish the utility of scintigraphy for measuring airway clearance, we performed additional evaluations and restudied mice with aerosol inhalation to determine reproducibility of the delivery technique and clearance within a given animal. Figure 4 presents the mean retention data of $^{99m}$Tc-SC in the airway of four mice over 6 h and at the final time point ~24 h after aerosol delivery. Animals were evaluated on two separate study days (with a 7-day interval between study days). The overlap of the mean activity time plots supports the potential utility of the methodology for restudy of a population of animals as their own controls, a study design frequently used in longitudinal studies and evaluations of mechanisms that impact on mucociliary function (9, 10).

Our investigation is not easily comparable to earlier studies of particle clearance in small rodent models because, in these investigations, the emphasis was placed on long-term, i.e., alveolar clearance, mechanisms (16, 23, 24). In these earlier studies, radiotracer activity was measured with single sodium iodide crystal detectors and whole body counting or within excised tissue and collected excreta from animals serially killed. Snipes et al. (23) estimated that the tracheobronchial region cleared with a half-time of 4.1 h, whereas mechanical clearance of the alveolar region was represented by a two-component exponential expression that yielded an initial biological half-time of 35 days. In a mouse deposition study by Raabe et al. (20), lung retention was also determined using sodium iodide probes and collection of excreta, and the activity that cleared in the initial 20 h after deposition was considered to represent the tracheobronchial fraction. In the Raabe study for an ~1-μm diameter aerosol that was inhaled with a head-only exposure system, 42% of the initial lung burden cleared during the initial 20-h period after deposition, a fraction quite comparable to the 43% that we observed for a nose-only inhalation system and a slightly smaller-diameter aerosol.

In summary, we have developed a methodology that uses scintigraphy to measure in vivo the clearance of insoluble SC particles delivered to the lower respiratory tract of the mouse. For a submicrometer-sized aerosol a nose-only aerosol inhalation technique appeared to be optimum for maximizing deposition of the particles onto airway surfaces (cleared by mucociliary function). Scintigraphy permitted noninvasive visualization of deposited activity within the respiratory tract and afforded precise measurement of particle clearance from lung regions. Delivery techniques did not lead to inflammation of pulmonary tissues, were repeatable, and produced reproducible airway clearance kinetics for insoluble particles.

The authors express their appreciation to Dr. Marsha Wills-Karp for laboratory support and Malik Richardson for help in formatting the artwork.

This research was supported by National Heart, Lung, and Blood Institute Grant HL-62641 and National Institute of Environmental Health Sciences Grant ES-03810 (Washington, DC).

REFERENCES


AIRWAY MUCOCILIARY TRANSPORT IN MICE


